IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Mountz, *et al.*	§	**ART UNIT:**
	§	**1632**
FILED: May 15, 1998	§	
	§	**EXAMINER:**
SERIAL NO.: 09/079,834	§	**Wehbe, A.M.S.**
	§	
FOR: Fas Ligand Expressing Antigen Presenting Cells For Tolerance Induction	§	**DOCKET:**
	§	**D6005**

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

ATTENTION: Board of Patent Appeals and Interferences

TRANSMITTAL OF APPEAL BRIEF

Dear Sir:

Enclosed please find an Appeal Brief for the above-referenced patent application. The Commissioner is hereby authorized to charge $250 to the credit card indicated on the attached form PTO-2038.

Respectfully submitted,

Date: Jan 19, 2006

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Alexandria, VA 22313

APPEAL BRIEF

This Brief is in furtherance of the Notice of Appeal mailed in this case on March 23, 2005. The fees required under 37 C.F.R. §41.20(b)(2) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

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Respectfully submitted,

Date: Jan 19 2006

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INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is The University of Alabama at Birmingham Research Foundation, the Assignee, as evidenced by an Assignment recorded in the Patent and Trademark Office at Reel 011157, Frame 0104 on September 28, 2000.

II. RELATED APPEALS AND INTERFERENCES

Appellant is aware of four prior appeals of the present invention. In the Appeal Brief mailed April 6, 2000, claims 1, 3-6 and 16 were appealed. Claim 17 was rejected under 35 U.S.C. §102 for being anticipated by **Bellgrau** *et al* and claims 1 and 3-6 were rejected under 35 U.S.C. §103(a), for being obvious over **Bellgrau** *et al* in view of **Süss** *et al* and **Bellgrau** *et al* in view of **Schuler** *et al*. Further, claim 16 was rejected under 35 U.S.C. §103(a) for being obvious over **Bellgrau** *et al* in view of **Süss** *et al* and **Bellgrau** *et al* in view of **Schuler** *et al*. However, in the Office Action mailed June 19, 2000, the Examiner withdrew the finality of rejection of the Office Action mailed October 29, 1999 which was addressed in the Appeal Brief mailed April 6, 2000.

Further, in the Appeal Brief mailed February 19, 2003, claims 1, 3-6, 8, 9 and 16 were appealed. Claims 1, 3-6, 8, 9 and 16 were rejected under 35 U.S.C §112, first paragraph for lack of enablement. However, in the Office

Communication mailed May 6, 2003, the Examiner stated that the Appeal Brief mailed February 19, 2003 was defective for failure to comply with one or more provisions of 37 C.F.R. 1.192(c) since the Appellants had not presented arguments in support of their position of one or more claims not standing or falling together.

In the Appeal Brief mailed May 27, 2003, claims 1, 3-6, 8, 9 and 16 were appealed. Claims 1, 3-6, 8, 9 and 16 were rejected under 35 U.S.C §112, first paragraph for lack of enablement. However, in the Office Communication mailed August 12, 2003, the Examiner stated that the Appeal Brief mailed May 27, 2003 did not comply with one or more provisions of 37 C.F.R. 1.192(c) since the Appellants had not presented any specific argument as to why claim 16 did not stand or fall with claims 1, 3-6, 8 and 9.

In the Appeal Brief mailed August 28, 2003, claims 1, 3-6, 8, 9 and 16 were appealed. Claims 1, 3-6, 8, 9 and 16 were rejected under 35 U.S.C §112, first paragraph for lack of enablement. In this Appeal Brief, the Appellant had presented arguments as in support of their position of one or more claims not standing or falling together. However, in the Office Action mailed May 18, 2004, the Examiner withdrew the previous rejection in view of new grounds of rejection.

III. STATUS OF CLAIMS

Originally claims 1-17 were filed with this Application. Claims 10-15 were withdrawn from consideration. Claims 2, 7 and 17 were canceled and claims 1 and 16 were amended. Subsequently claim 16 was canceled and claims 1 and 8 were amended. Pending claims 1, 3-6 and 8-9 are being appealed, of which claim 1 is an independent claim.

IV. STATUS OF AMENDMENTS

Subsequent to the Final Office Action mailed October 29, 1999, claim 17 was canceled and new claims 18 and 19 were added. These new claims were not entered since they required a separate search as stated in the Advisory Action mailed December 8, 1999.

The Office Action mailed June 19, 2000 stated that the finality of rejections in the Final Office Action mailed October 29, 1999 were withdrawn. Although claims 1 and 16 were amended in response to this Office Action, no amendments were made in response to the Final Office Action mailed March 23, 2001. Furthermore, although claims 1 and 8 were amended and claim 16 and withdrawn claims 10-15 were canceled in response to Office Action mailed May 18, 2004, no amendments were made in response to Final Office Action mailed September 23, 2004. All pending claims are shown in Appendix A.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The instant invention is directed to a method of inducing systemic tolerance to an antigen in an individual in need of such treatment (claim 1). This method comprises the step of administering to the individual antigen presenting cells which (1) express high levels of Fas ligand resulting from co-infection with AdLoxFasL and AxCANCre adenoviruses, (2) do not express Fas and (3) express the antigen, where the antigen presenting cells induce apoptosis of Fas-positive T cells directed towards the antigen resulting in the induction of systemic tolerance to the antigen. The antigen recited in this method may be selected from the group consisting of the adenovirus antigen, a viral antigen, an adeno-associated viral antigen, an autoantigen and an alloantigen (claim 3).

The individual benefiting from this method may have an autoimmune disease (claim 4). Furthermore, the autoimmune disease may be diabetes, multiple sclerosis, rheumatoid arthritis, thyroiditis, Grave's disease or systemic lupus erythematosus (claim 5). Additionally, the individual benefiting from this method may have had an organ transplant (claim 6). The method may further comprise the step of delivering in vitro to the antigen presenting cells a gene, such as crmA, to inhibit apoptosis (claim 8 and 9).

The instant invention discloses a method of inducing systemic tolerance to a viral or an alloantigen using Fas negative antigen presenting cells that are engineered to express Fas ligand and the antigen of interest. These

antigen presenting cells induced apoptosis of Fas-positive T cells directed towards the antigen through the Fas ligand-Fas interaction which resulted in induction of systemic tolerance to the antigen (page 9, line 3-page 10, line 5). The instant invention also teaches the usefulness of such a method in the treatment of graft rejection and autoantigen-specific autoimmune diseases (page 21, lines 16-18).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 1, 3-6 and 8-9 stand rejected under 35 U.S.C. §112, first paragraph for lack of enablement.

VII. GROUPING OF CLAIMS

The rejected claims stand or fall together.

VIII. ARGUMENT

Rejection Under 35 U.S.C. §112, first paragraph

Claims 1, 3-6 and 8-9 remain rejected under 35 U.S.C. §112, first paragraph for lack of enablement. Applicant respectfully traverses this rejection.

In the Advisory action mailed March 31, 2005, the Examiner has maintained the rejection of claims 1, 3-6 and 8-9 under 35 U.S.C. §112, first paragraph for lack of enablement in the specification for inducing systemic tolerance using antigen presenting cells other than fas-negative peritoneal macrophages. The Examiner contends that "antigen presenting cells" is a broad term and encompasses cells that express MHC class I or MHC class II (Final Office Action mailed May 18, 2004 and Advisory Action mailed March 31, 2005). The Examiner states that although the Applicant prefered to label MHC class I cells as target cells in their response to the Final Office Action mailed May 18, 2004, these cells are antigen presenting cells since they process and present antigens in context of MHC class I to T cells.

While pointing to Applicant's definition of professional and non-professional antigen presenting cells in the response to the Final Office Action mailed May 18, 2004, the Examiner states that it supports the Office's position that the specification is not enabling for all antigen presenting cells. Further, the Examiner states that the evidence of **Hoves** *et al* reference was not entered since the arguments directed to **Hoves** *et al* were not found persuasive. Additionally, the Examiner states that Applicant's argument that cells referred to

in **Restifo** and **Kang** were not antigen presenting cells is refuted by Applicant's own statement on page 5 of the response that fibroblasts, pancreatic beta cells and epithelial cells are antigen presenting cells.

Applicant would like to respectfully point out that the labeling of MHC class I-expressing cells as target cells is not the Applicant's preference but an accepted concept in the art as taught by the reference cited by the Applicant in the response to Final Office Action (see attached excerpt from Immunology, 3rd edition, Janis Kuby, pg. 253. line 1-31). The reason being that the antigens presented in context of MHC class I molecule are intracellular antigens such viral antigens, tumor antigens, etc that are normally presented to cytotoxic CD8+ T cells. When recognized by these cytotoxic T cells, the cells expressing such antigens are destroyed via perforin mediated or Fas-Fas ligand interaction by the cytotoxic T cells. Hence, such cells are labeled as target cells.

Further, with regards to Applicant's definition of professional and non-professional antigen presenting cells indicating that the specification is not enabling for all antigen presenting cells, Applicant had used this definition and **Hoves** *et al* (*J Immun.*, 2003, 170: 5406-5413) to point out the type of cells that are commonly used as antigen presenting cells in the art. The citation of **Hoves** *et al* in the response also illustrated how the teachings of the instant specification was sufficient to enable one to practice the claimed method with antigen presenting cells other than the one used in the instant invention.

The role played by antigen presenting cells in determining whether an immune response is immunogenic or tolerogenic is also well-known in the art

and described in the instant specifcation. For example, an autoimmune response develops as a result of activation of autoreactive T cells which in turn are activated due to inappropriate antigen presentation by certain MHC molecules. Thus, the interaction between T cells and antigen presenting cells plays an important role in the initiation of such a response (page 58, lines 5-19). It is well-established in the art that CD4+ T cells are primary mediators of autoimmune disease since the development of this disease depends on the balance Th1/Th2 response. Thus, treatment of this disease would require elimination of CD4+ T cells.

Further, direct antigen presentation is a major component of allogeneic T cell activation and allograft rejection. The graft rejection occurs due to T cell response to alloantigens released from the grafted tissue that are carried on the antigen presenting cells. The significance of CD4+ T cells in the graft rejection is well-established in the art since these cells can either induce delayed type hypersensitivity response or cytotoxic T cell response, both of which results in graft rejection. Thus, prevention of such rejection would require elimination of the CD4+ T cell.

With regards to gene therapy, an immune response to the virus carrying the protein or the gene therapy protein itself results in failure of the gene therapy. Such a failure can be prevented by inducing peripheral T cell tolerance that is specific for the viral vector (page 7, line 1-page 8, line 4). In general, the present invention discloses a method of using genetically modified Fas ligand-expressing antigen presenting cells to induce apoptosis of Fas-positive antigen-

specific T cells, thereby inducing systemic tolerance to the antigen. The instant specification teaches that such a method will be useful in the treatment of autoimmune disease, prevention of graft rejections and in the use of gene therapy (page 1, line 19-page 20, line 2) and discussed the mechanism in each of these conditions (see supra).

The instant invention used macrophages such as peritoneal macrophages to demonstrate the induction of systemic tolerance. As is known in the art, macrophages belong to a class of professional antigen presenting cells. Examples of other cells that belong to this group are dendritic cells and B cells. These cells express MHC class II molecules and co-stimulatory molecules either before or after they are activated. Such MHC class II expressing cells are then recognized by CD4+ T cells (T helper cells). The instant specification teaches that the antigen presenting cells used in the claimed method were Fas negative cells which ensured protection of these cells from apoptosis once they expressed Fas ligand. These cells were also infected with AdLoxPFasL and AxCANCre adenoviruses that enabled high levels of Fas ligand expression in addition to the control of this expression (Example 19). Additionally, these antigen presenting cells were loaded with the antigen that could be processed and presented to T cells, which is necessary to induce Fas-ligand mediated apoptosis of T cells. The instant specification provides ample guidance to one skilled in the art to construct the adenoviral vectors discussed above (Example 2) and the experiments that can be performed to assess the induction of tolerance *in vivo* by these Fas-ligand expressing vectors (Examples 16-23). Additionally, the instant

specification teaches that the Fas-ligand expressing antigen presenting cells migrated to the lymphoid organs and induced apoptosis of T cells (Example 18) and that there was no liver damage associated with the systemic administration of these antigen presenting cells. Thus, the instant specification outlines the characteristics of antigen presenting cells that would be useful in the claimed method to be (1) Fas-ligand expressing; (2) Fas negative and (3) expressing the antigen.

Further, in the Final Office Action mailed September 23. 2004, the Examiner had cited several references such as **Restifo**, **Kang** *et al*, **Seino** *et al*, **Muruve** *et al* and **Zhang** *et al* to illustrate unpredictability in the art of using any antigen presenting cells since the antigen presenting cells described in these references induced inflammation in vivo. In response, Applicant pointed out the differences in the characteristics of apoptosis and necrosis that are two different types of cell death which result in different effects (Immunology, 3rd Edition, Janis Kuby, page 53, col. II, last para-page 54, col.I; Table 3-2). For instance, apoptosis would result in cell death and no inflammation while necrosis would result in cell death with induction of inflammation. In this context, since the claimed method recited that the Fas-ligand expressing antigen presenting cells would induce apoptosis of Fas-positive T cells and thereby induce systemic tolerance to the antigen, one skilled in the art would not regard Fas-ligand expressing antigen presenting cells that induced inflammation such as islet cells, fibroblasts, epithelial cells and tumor cells to be useful in the claimed method. Thus, the Applicant did not state that these cells were not antigen presenting

cells but by stating that "one of ordinary skill in the art would not ordinarily regard them as antigen presenting cells taught in the instant invention" meant that one of ordinary skill in the art would not regard them as antigen presenting cells that would be useful in context of the instant invention.

Applicants respectfully submit that "the specification need not contain an example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without undue amount of experimentation. Lack of a working example, however is a factor to be considered, especially in a case involving an unpredictable and undeveloped art. But because only an enabling disclosure is required, Applicant need not describe all actual embodiments" (M.P.E.P. 2164.02). In the instant case, the art of using antigen presenting cells is well-developed and the instant specification provides ample teaching for one skilled in the art to determine the right type of the antigen presenting cell that would work in the claimed method to produce the claimed effect.

Further, the test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosure in the patent coupled with the information known in the art without undue experimentation (M.P.E.P. 2164.01). Based on the detailed description of the in vivo effects of Fas ligand-expressing antigen presenting cells disclosed in the instant specification, Applicant submits that the instant specification has provided sufficient enablement for using the antigen presenting cells to induce T cell tolerance in humans. Thus, the scope of the claimed invention is commensurate with the

enablement provided. Accordingly, Applicant respectfully request withdrawal of rejection of claims 1, 3-6 and 8-9 under 35 U.S.C. §112, first paragraph.

Respectfully submitted,

Date: Jan 19, 2006

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CLAIMS APPENDIX

1. (previously presented) A method of inducing systemic tolerance to an antigen in an individual in need of such treatment, comprising the step of:

administering to said individual antigen presenting cells which (1) express high levels of Fas ligand resulting from co-infection with AdLoxFasL and AxCANCre adenoviruses, (2) do not express Fas and (3) express said antigen, wherein said antigen presenting cells induce apoptosis of Fas-positive T cells directed towards antigen resulting in said induction of systemic tolerance to said antigen.

2. (canceled).

3. (original) The method of claim 1, wherein said antigen is selected from the group consisting of the adenovirus antigen, a viral antigen, an adeno-associated viral antigen, an autoantigen and an alloantigen.

4. (original) The method of claim 1, wherein said individual has autoimmune disease.

5. (original) The method of claim 4, wherein said autoimmune disease is selected from the group consisting of diabetes, multiple sclerosis, rheumatoid arthritis, thyroiditis, Grave's disease, systemic lupus erythematosus.

6. (original) The method of claim 1, wherein said individual has had an organ transplant.

7. (canceled).

8. (previously presented) The method of claim 1, further comprising the step of delivering *in vitro* to said antigen presenting cells a gene to inhibit apoptosis.

9. (original) The method of claim 8, wherein said gene to inhibit apoptosis is crmA.

10-17. (canceled).

EVIDENCE APPENDIX

Kang et al, *Trans Proceed*,
30: 538 (1998)

Restifo et al., *Nat Med*,
6(5):493-495 (2000)

Seino et al., *Transp Proceed*,
29:1092-1093 (1997)

Zhang et al., *J Virol*,
72(3):2484-2490 (1998)

Muruve et al., *Human Gene Ther.*,
8:955-963 (1997)

Hoves et al, *J Immunol*,
170: 5406-5413 (2003)

Janis Kuby., *Immunology (3rd Edn)*,
Page 253: lines 1-31
page 53, col. II, last para-page 54, col.I
Table 3-2

ELSEVIER

Fas Ligand Expression on Islets as Well as Multiple Cell Lines Results in Accelerated Neutrophilic Rejection

S.-M. Kang, Z. Lin, N.L. Ascher, and P.G. Stock

FAS LIGAND (FasL, CD95L)[1] has been reported to confer immune privilege to the anterior chamber of the eye[2] as well as rodent testis,[3] presumably by causing apoptosis of Fas (CD95) bearing T lymphocytes. One group has reported that syngeneic myoblasts engineered to express FasL could protect allogeneic islets when cotransplanted under the kidney capsule.[4] We have previously shown, however, that FasL expression directly on islets via an adenoviral vector leads to accelerated rejection of the islets that is mediated by neutrophils.[5] This neutrophilic destruction was dependent on Fas expression on host tissue but not on the islets and did not require the presence of T or B lymphocytes. In addition, transgenic mice expressing FasL on islet β cells developed spontaneous diabetes, with neutrophilic infiltration of the islets.

To test whether this unexpected neutrophilic response to FasL was specific for islets, we expressed FasL on a variety of cell lines and tested the host response to the cells after injection of 1×10^6 cells subcutaneously or under the kidney capsule. The cell lines tested include NIH 3T3 (murine fibroblast), L5178 (murine lymphoma), Cos-7 (monkey epithelial), P815 (murine mastocytoma), and BTC20 (murine insulinoma). All cell lines expressing FasL induced a neutrophilic infiltrate, although the intensity of the neutrophilic response varied significantly between cell lines. FasL expressing P815 cells and L5178 cells did not grow in syngeneic hosts, while the parental cells formed large, lethal tumors. Myoblasts were not protected from this neutrophilic response, as FasL expressing myoblasts were also destroyed in syngeneic mice by a neutrophilic infiltrate.[6] Moreover, syngeneic FasL expressing myoblasts were found to accelerate rather than delay the rejection of allogeneic islets when co-injected under the kidney capsule. Thus, FasL induces a destructive neutrophilic infiltrate under a variety of conditions. The molecular determinants of this response and the precise role of FasL in immune privilege will need to be identified prior to the development of FasL-based immunotherapy.

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2. Griffith TS, Brunner T, Fletcher SM, et al: Science 270:1189, 1995
3. Bellgrau D, et al: Nature 377:630, 1995
4. Lau HT, Yu M, Fontana A, et al: Science 273:109, 1996
5. Kang S, et al: Nature Medicine 3:738, 1997
6. Kang S, Hofmann A, Le D, et al: Science 278:1322, 1997

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Supported in part by a Hefne Grant to PGS. SMK is a physician-postdoctoral fellow of the Howard Hughes Medical Institute.

Address reprint requests to Dr P. Stock, UCSF Transplant Division, Box 0116, 513 Parnassus Ave, San Francisco, CA 94143.

0041-1345/98/$19.00
PII S0041-1345(97)01396-1

Based on early studies, it was hypothesized that expression of Fas ligand (FasL) by tumor cells enabled them to counterattack the immune system, and that transplant rejection could be prevented by expressing FasL on transplanted organs. More recent studies have indicated that the notion of FasL as a mediator of immune privilege needed to be reconsidered, and taught a valuable lesson about making broad conclusions based on small amounts of data.

Not so Fas: Re-evaluating the mechanisms of immune privilege and tumor escape

NICHOLAS P. RESTIFO

The history of science is full of ideas that are at once so elegant and so obvious that they take root in our imaginations, despite relatively weak support by available data. One such idea now in the tumor immunology community is that tumor cells use a molecule called Fas ligand (FasL) to counter-attack the immune system[1]. Despite substantial evidence to the contrary, this molecule is thought by some to induce the death and elimination of T lymphocytes that enter the tumor bed, thereby granting the tumor immune-privileged status. But this idea is based on inference: all well-controlled experiments in which FasL expression is induced in a tumor or tissue, either through use of transgenic mice or by transfection or transduction of transplanted cells, have shown that the tissue is rapidly rejected, without evidence of immune privilege.

The original idea was logical. FasL expressed on tissues would engage the Fas receptor expressed on the surfaces of immune cells, causing them to undergo programmed cell death (Fig. 1). The idea that FasL (also known as CD95 ligand or APO-1 ligand) could help tumors counter-attack the immune system has its origins in the field of transplantation. However, several early papers containing evidence initially thought to support the hypothesis that FasL could grant immune-privileged status have now been withdrawn or refuted.

The concept was brought to popular attention in a *News and Views* article published in *Nature* in October 1995 (ref. 2). The author summarized findings demonstrating that Sertoli cells expressing FasL could be transplanted into allogeneic mice and concluded, based on the data, that the interaction of Fas and its ligand was at the heart of immune privilege[3]. Another group then reported that transplanted tissue could be protected from rejection if one simply surrounded tissue (in this case the insulin-producing Islet cells of the pancreas) with myoblasts expressing FasL (ref. 4). Fas–FasL interactions were also reported to be fundamental to immune privilege in the eye[5]. The implications of these findings were great: Graft rejection could be prevented if cells or organs were transfected with FasL before transplantation.

Shortly after these initial publications, two sets of data were published that had a substantial effect on the tumor immunology community, catalyzing a flurry of research activity based on these findings. The first described FasL expression by colon tumor cells, claiming that this induced cell death of Jurkat cells (a human T-cell leukemia line)[6]. The second described a similar set of results using melanoma[7]. The latter demonstrated a remarkable consistency of FasL expression in tumor cell samples: All of the 10 melanomas tested expressed FasL. Moreover, it showed that this FasL was functional. The authors also claimed that infiltrating T cells could only be found proximal to FasL-positive lesions of human metastatic melanoma, a finding at odds with many other observations of tumor-infiltrating lymphocytes. Furthermore, the authors reported that the highly virulent mouse melanoma cell line, B16, was FasL-positive. The authors asserted that FasL expression by B16 caused rapid tumor formation.

After those reports, it seemed that the evidence on the function of FasL in transplantation, autoimmunity and tumor escape was clear and compelling. But some other experimental data did not fit the newly established paradigm of FasL as the enforcer of immune privilege. When Allison *et al.* used transgenic mice expressing FasL on their islet β cells for transplantation, they found that rather than being the solution to the transplantation immunologist's rejection problem, expression of FasL caused a more rapid rejection of islet cells accompanied by a "granulocytic infiltration" (ref. 8). Kang *et al.* used an entirely different approach to test the same question, using adenoviruses to confer FasL expression on islet cells. They also found accelerated rejection accompanied by "massive neutrophilic infiltrates" (ref. 9). Similar results were obtained using other cells[10,11]. Over the two years that followed, Seino *et al.* would publish many papers testing a possible use for FasL in transplantation, but their data refuted the idea that conferring FasL expression on transplants would be therapeutically useful as originally described[6,12–15]. Instead, ectopic FasL expression caused rapid rejection and profound inflammation with abscess formation.

In vitro and *in vivo* experiments with tumor cells also contradicted the original hypothesis that FasL mediated an immune counter-attack. Some investigators claimed that they could not find FasL on the surfaces of melanoma cells[16,17], and when they transfected tumor cells with the gene encoding FasL, they did not detect 'tumor escape'. Instead, there was rapid tumor rejection in many experimental tumor systems (including the B16 melanoma)[10,17]. As with other FasL-based transplantation experiments, rejected tumors sites were infiltrated with granulocytes that coalesced into abscesses. Soluble FasL can, in part, abrogate the inflammatory effects of membrane-bound FasL (ref. 18).

So why did FasL trigger inflammation? A definitive answer to this question is unknown. However, it is clear that Fas signaling activates a caspase cascade. One consequence of this cascade is the activation of interleukin-1β-converting enzyme (ICE), also known as caspase 1 (ref. 19). As its name indicates, ICE is capable of cleaving interleukin-1β from its inactive form into its active form[20]. Once activated, interleukin-1β is a potent pro-inflammatory cytokine. ICE also cleaves and activates IL-18: the disabling of ICE is a strategy used by poxviruses to evade immune destruction[21].

Although some early authors sought to retract their earlier statements, others 'stuck to their guns'. It was important for trans-

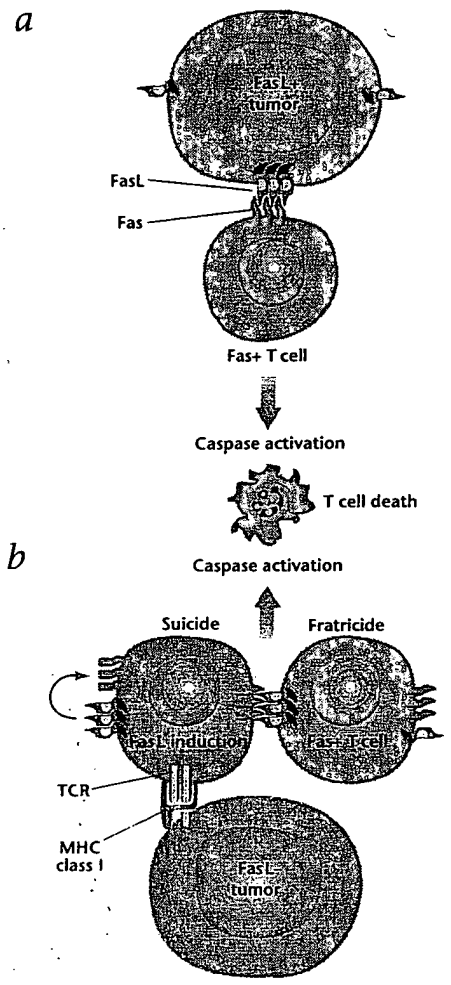

Fig. 1 FasL-induced T cell death. ***a***, Early experiments indicated that FasL, expressed on tumor cells, interacts with its receptor, Fas, which is expressed on invading T cells. This interaction would trigger T-cell death, through caspase activation, and grant the tumor immune privilege. ***b***, Recent studies have shown that FasL is expressed by T lymphocytes after tumor recognition and T cell activation. T cells then kill themselves ('suicide') and each other ('fratricide') through the same caspase-based mechanism.

plant immunologists to correct their original mistakes, as transplants engineered to express FasL could prove to be disastrous, leading to rapid rejection and abscess formation rather than increased tissue engraftment. Vaux, who wrote one of the earliest and clearest descriptions of FasL as the 'enforcer' of immune privilege, took the unusual step of retracting his *News and Views* piece after his own lab found inflammation, not immunosuppression[2].

The continued proliferation of erroneous ideas about FasL may have been due in part to technical problems hindering early research. A principal problem involved the antibodies used in the early reports. A polyclonal antibody against human FasL (C-20), produced by Santa Cruz Biotechnologies, was not highly specific[22], leading to the publication of false-positive results on a least a half-dozen different occasions by the time its cross-reactivity was discovered in 1998, and in several reports by others since then[23]. Compounding the problem may be the fact that many other polysera were generated in a similar way[24], but were not tested in 1998 report[22]. An underlying and ongoing problem is the use of polysera made by injecting animals with peptides. Although the generation of polyclonal antibodies is straightforward, a complete characterization of antibody specificity is difficult and fraught with pitfalls. Another widely used reagent, a mouse monoclonal antibody (clone 33) from Transduction Laboratories, was also reported to be nonspecific for FasL (ref. 25).

Other possible confounding variables include the use of non-intron-spanning PCR primers without proper controls[7,26], contamination of fresh tumor samples with lymphocytes (which can express large amounts of FasL) and problems with functional assays. For example, several studies involved T-cell targets that were themselves capable of expressing FasL, such as Jurkat cells. When target cell death was found and blocked with antibodies against FasL, researchers were not always able to verify that the cell death was induced by FasL expression on tumor cells and not by induced expression of FasL on T cells[26]. Thus, the devil really was in the details.

One consequence of these misunderstandings, for tumor immunology, was that researchers have been side-tracked in determining the true biological function of Fas and FasL as mediators of cytotoxicity[27] and as central mediators of activation-induced cell death[28]. Thus, it is the case that Fas-FasL interactions can cause T cell death and this death is important in the induction of tolerance, immune homeostasis and lymphocyte effector functions. Indeed, it has been confirmed that melanoma-specific T lymphocytes undergo apoptotic death after the major-histocompatibility-complex-restricted recognition of tumor cells, and T-cell death can be blocked by the addition of a specific antibodies against Fas (ref. 29). However, contrary to the prevailing view that tumor cells cause the death of anti-tumor T cells by expressing FasL, it is now apparent that in most cases, FasL is expressed by T lymphocytes upon activation after tumor cell recognition, causing them to kill themselves ('suicide') and each other ('fratricide') (Fig. 1)[16,29,30]. When FasL is expressed ectopically with the goal of inducing the death of T lymphocytes, researchers must consider the resultant caspase cascade and the potential for the consequent activation of an innate immune response.

Hindsight is always perfect, and it is now possible to view initial mistakes and contradictory data regarding the function of FasL in a new light. A tantalizing new idea, featured prominently in the literature, can rapidly spread through the scientific community. Once a widely believed hypothesis is re-evaluated, 'cool' negative results may not receive the same attention as 'hot' positive results. Furthermore, corrections and retractions, as in politics, are often hidden in less-than-obvious places, such as during the question-and-answer periods of lectures, or in abstracts or posters at scientific meetings. It may be true that science is a self-correcting enterprise, and theories come and go, but even in this age of rapid communication, the progress of truth can be glacially slow. Scientific researchers and journal editors must be ever-wary of elegant and intuitively obvious ideas that are only weakly supported by available data.

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Adenovirus-Mediated Expression of Fas Ligand Induces Hepatic Apoptosis after Systemic Administration and Apoptosis of *Ex Vivo*-Infected Pancreatic Islet Allografts and Isografts

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ABSTRACT

Fas ligand (FasL) mediates apoptosis of Fas-bearing cells and is expressed on a limited number of tissues, predominantly activated T lymphocytes. We describe the construction and biological activity of a replication-deficient type-5 adenovirus encoding murine FasL under the control of the cytomegalovirus (CMV) promoter (adCMV-FasL). *In vitro,* Jurkat cells undergo apoptosis when co-incubated with adCMV-FasL-infected COS cells. Systemic administration of adCMV-FasL to Wistar rats or DBA/2J mice results in widespread hepatic apoptosis and death in a dose-dependent manner within 72 hr, an effect not seen in *lpr* mice, or animals administered equivalent doses of adCMV-βgal. Murine pancreatic islets also undergo apoptosis when infected *ex vivo* with adCMV-FasL, resulting in uniform primary nonfunction when transplanted into syngeneic or allogeneic diabetic recipients. These results indicate that adCMV-FasL is a potentially useful tool to study Fas/FasL biology.

OVERVIEW SUMMARY

Expression of Fas ligand (FasL) has recently been associated with immune privilege, and has been shown to prolong pancreatic islet allograft survival when expressed on co-transplanted syngeneic myoblasts. To explore the possibility of using Fas-mediated apoptosis as a tool to destroy activated T cells in an organ transplant setting, we have generated a replication-deficient type-5 adenovirus encoding murine FasL under the control of the cytomegalovirus (CMV) promoter (adCMV-FasL). In this paper, we demonstrate that adCMV-FasL is a potent inducer of apoptosis *in vitro* and *in vivo*. Furthermore, *ex vivo* infection of pancreatic islet isografts and allografts with adCMV-FasL resulted in widespread apoptosis of the islets and dramatically reduced survival in a murine pancreatic islet transplant model. These data suggest that adCMV-FasL may be a useful tool to further study Fas/FasL biology, but its ability to induce apoptosis of transduced grafts effectively is detrimental in the transplant setting.

INTRODUCTION

Fas ligand (FasL) is a type II membrane protein belonging to the tumor necrosis factor family whose interaction with its receptor Fas (CD95) mediates apoptosis of many Fas-bearing cells. Whereas most cells express varying levels of Fas, FasL is expressed predominantly on activated T lymphocytes. The Fas/FasL interaction plays a significant role in immune regulation as well as cytotoxic T lymphocyte-mediated cell killing (Nagata and Golstein, 1995).

Induction of FasL on T lymphocytes and T cell lines results in cell death by apoptosis (Dhein *et al.*, 1995). In addition, activation of Fas via soluble FasL or activating anti-Fas antibodies results in apoptotic cell death in both lymphoid and certain nonlymphoid tissues (Ogasawara *et al.*, 1993; Rensing-Ehl *et al.*, 1995). In contrast, FasL expression in certain tissues such as testis or cornea creates immune privilege (Bellgrau *et al.*, 1995; Griffith *et al.*, 1959). Lau *et al.* recently demonstrated in a murine pancreatic islet transplant model that FasL-expressing

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myoblasts co-transplanted with islet allografts resulted in prolonged survival (Lau *et al.*, 1996). As we learn more about Fas/FasL, a potential role for the manipulation of this pathway has emerged in other diseases such as cancer and autoimmune disorders.

Replication-deficient adenoviruses are efficient vectors for transducing replicating and nonreplicating eukaryotic cells. We describe the generation of a FasL-expressing adenovirus vector (adCMV-FasL) and the biological effects of FasL expression *in vitro, in vivo,* and in a pancreatic islet transplant model.

MATERIALS AND METHODS

Animals

Male DBA/2J (H-2^d), C3H/HeJ (H-2^k), C57BL/6 (H-2^b), B6A/F1 (H-$2^{b/k,d}$), and MPJ-MRL-*lpr* (H-2^k) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed under standard conditions. Mice were used for the described experiments at 8–14 weeks of age. Male Wistar rats were purchased from Charles River (Wilmington, MA). The rats ranged from 125 to 135 grams in weight.

Construction and purification of adCMV-FasL

The 943-bp cDNA *Xba* I fragment containing murine FasL (a generous gift from S. Nagata) was inserted into the *Xba* I site of the plasmid pACCMV.pLpA (Gluzman *et al.*, 1982), cotransfected with pJM17 into 293 cells, and plaque purified as previously described (Becker *et al.*, 1994). Plaques were screened for recombinant adenoviruses using the polymerase chain reaction (PCR) and primers encoding murine FasL and adenoviral fiber protein. Positive recombinants were identified through detection of both FasL and adenoviral fiber protein gene products with PCR. Viral titer was measured by standard plaque assay using 293 cells.

Reverse transcription and PCR

Total RNA from liver, pancreatic islets, and 293 cells was isolated using the RNeasy procedure (Qiagen Chatsworth, CA). Reverse transcriptions were performed by oligo$(dT)_{12-18}$ priming (GIBCO-BRL, Grand Island, NY) and using Moloney murine leukemia virus (Mo-MLV) reverse transcriptase (GIBCO-BRL) in deoxyribonuclease I (GIBCO-BRL) -treated samples. Deoxyribonuclease-treated samples without reverse transcriptase were used as negative controls. Each PCR used 0.1 ng of cDNA or 2 μl of 1:100 diluted viral supernatant, 4 ng/μl of each primer, 0.25 units of *Taq* polymerase (Promega, Madison, WI), 150 μM of each dNTP, 3 mM of $MgCl_2$, reaction buffer, and water to a final volume of 50 μl and was covered with mineral oil. The sequences for human Fas primers were: sense, 5′-CACTTCGGAGCATTGCTCAACA-3′; antisense, 5′-TATGTTGGCTCTTCAGCGCTA-3′ (Cheng *et al.*, 1994). The sequences for murine Fas primers were: sense, 5′-ATGCTGTGGATCTGGGCTGTC-3′; antisense, 5′-TCACTCCAGACATTGTCCTTCA-3′ (Larsen *et al.*, 1995). The sequences of the murine FasL primers were: sense, 5′-GCCGACCGCAGCCCGCATCCAGC-3′; antisense, 5′-CGCCGCCACTGGTGTGGGCC-3′ (Takahashi *et al.*, 1994). The sequences of the primers for adenoviral fiber protein were: sense, 5′-CGCCGCACCTCTAATGGTCG-3′; antisense, 5′-CCTGGACCAGTTGCTACGGTC-3′. The sequences of the *E. coli* β-galactosidase primers were: sense, 5′-GCCGACCGCAC-GCCGCATCCAGC-3′; antisense, 5′-CGCCGCGCCACTGGTGTGGGCC-3′ (Csete *et al.*, 1995).

PCR was performed using 1 min for denaturation at 94°C, 1 min annealing at 58°C, and 1 min for extension at 72°C, for a total of 30 cycles. Thirty-five cycles were used for the amplification of adenoviral fiber protein cDNA from tissues. The amplified products were separated on 1% agarose gels containing ethidium bromide and were visualized by ultraviolet illumination.

Protein electrophoresis and Western blotting

COS cells at 80% confluency in 100-mm plates were infected with approximately 10^9 pfu of adCMV-FasL or pFG140 (Microbix, Toronto, Canada) and incubated for 24 hr at 37°C, 5% CO_2. Cells were collected, centrifuged into a pellet, and lysed with 5% NP-40, 150 mM NaCl, 20 mM Tris, 1% leupeptin, 1% aprotinin, and 1% phenylmethyl sulfonyl fluoride (PMSF) for 15 min and then centrifuged at 14,000 rpm for 15 min at 4°C. A 6× SDS reducing buffer was added to the supernatant, and proteins were separated on a 10% SDS-polyacrylamide gel, and subsequently transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked in HEPES-buffered saline (HBS)/5% skim milk overnight, and then incubated with a 1:1,000 dilution of polyclonal rabbit anti-mouse Fas ligand antibody (Santa Cruz, Santa Cruz, CA) for 1 hr. After washing for 30 min with HBS/Tween, the membrane was incubated for 30 min with a 1:1,000 diluted horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham, Arlington Heights, IL). After another 30-min wash with HBS/Tween, proteins were detected using ECL system (Amersham).

Systemic administration of adCMV-FasL

On the day prior to viral administration, rats were injected intramuscularly (i.m.) with 130 mg/kg dexamethasone and 5 mg/kg diphenhydramine (both from Elkins-Sinn, Cherry Hill, NJ). Administration of these medications was repeated at the same dosage 2 hr prior to viral infusion. Eight rats were infused via the tail vein with 5×10^8, 1×10^9, or 6.8×10^9 pfu of adCMV-FasL or equivalent titers of adCMV-βgal (Herz and Gerard, 1993; Becker *et al.*, 1994). Fourteen DBA/2J mice were injected via the tail vein with 2×10^8, 4×10^8, or 1×10^9 pfu of adCMV-FasL or equivalent titers of adCMV-βgal. Three MPJ-MLR-*lpr* mice were injected with 1×10^9 pfu of adCMV-FasL. Animals were sacrificed at 24 and 72 hr, and the livers were harvested for histopathology and total RNA, or followed for survival.

Pancreatic islet isolation and transplantation

Pancreatic islets were isolated from DBA/2J or C3H/HeJ mice after collagenase (2 mg/ml) perfusion and digestion followed by discontinuous Ficoll gradient centrifugation (O'Connell *et al.*, 1993). Islets were infected in petri dishes containing RPMI-1640 media supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin. AdCMV-βgal or

adCMV-FasL were added to the culture at a multiplicity of infection (moi) of 20:1 or 2:1. Infection was carried out by incubating pancreatic islets with recombinant adenovirus for 90 min at 37°C, 95% humidity, and 5% CO_2. Islets were washed twice with complete RPMI 90 min after infection and then transplanted under the kidney capsule of mice previously rendered diabetic by intraperitoneal streptozotocin (Sigma, St. Louis, MO) injection at a dose of 250 mg/kg. DBA/2J islets were transplanted into syngeneic DBA/2J recipients, and C3H/HeJ or DBA/2J islets were transplanted into allogeneic C57BL/6 or B6A/F1 recipients, respectively. Approximately 300 islets were transplanted into syngeneic recipients, and approximately 500 were used in the allogeneic transplants. Tail vein blood glucose monitoring was performed at regular intervals to monitor islet graft function. Islet grafts were harvested at 24, 48, or 72 hr post transplant and processed for histopathology and total RNA. For *in vitro* analysis, after infection and washing, pancreatic islets were incubated in complete RMPI at 37°C, 95% humidity, 5% CO_2 for 6, 12, 24, or 48 hr and then processed for TUNEL staining or total RNA.

Detection of apoptosis

COS cells were infected with approximately 10^8 pfu of adCMV-FasL or pFG140 for 90 min at 37°C, 5% CO_2, washed twice with PBS, and incubated for 24 hr in fresh DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Jurkat cells (5×10^6) were added to adCMV-FasL- or pFG140-infected COS cells and incubated for 6 hr at 37°C, 5% CO_2. Jurkat cells were collected from the plates and labeled with fluorescein-conjugated dUTP using the In Situ Cell Death Detection Kit (Boehringer Mannheim, Indianapolis, IN). Cells were then analyzed using a FACStar PLUS flow cytometer and CellQuest software (Becton Dickinson). Tissues from animals treated with adCMV-FasL or adCMV-βgal were obtained from animals at predetermined intervals and cryopreserved in O.C.T. compound (Miles, Elkahart, IN). Tissue sections were stained with fluorescein-conjugated dUTP using the In Situ Cell Death Detection Kit and analyzed by fluorescence microscopy.

Immunohistochemistry

Islet grafts were obtained from animals at predetermined intervals and cryopreserved in O.C.T. compound (Miles). Frozen tissue sections (6–8 μm thick) were fixed for 10 min in acetone at −20°C. Sections were quenched in 0.3% H_2O_2 in methanol for 30 min, and then blocked with avidin/biotin and goat serum (Vector Laboratories, Burlingame, CA). Sections were then incubated with a 1:100 dilution of polyclonal rabbit anti-mouse Fas ligand antibody (Santa Cruz) for 2 hr at room temperature. After washing with PBS, sections were incubated with goat anti-rabbit IgG antibody for 30 min at room temperature, and then stained using the ABC protocol and DAB substrate (Vector Laboratories).

Histopathology and β-galactosidase histochemistry

Tissue blocks 2–4 mm thick were fixed in 4% paraformaldehyde/PBS at 4°C for 2 hr, after which tissue was washed for 24 hr in 30% sucrose/PBS and frozen in O.C.T. compound (Miles). Sections were cut and incubated for 10 min in 4% paraformaldehyde/PBS, washed three times with PBS and incubated at 37°C overnight with β-galactosidase (β-Gal) substrate solution containing 0.4 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Sigma, St. Louis, MO), 4 m*M* potassium ferricyanide, 4 m*M* potassium ferrocyanide, and 2 m*M* magnesium chloride. Snap-frozen tissue sections were cut and stained with Hematoxylin & Eosin.

Statistical analysis

All values are expressed as the mean ± SEM. Differences between the means were compared using Student's *t*-test.

RESULTS

Detection of FasL and apoptosis in vitro

Production of adCMV-FasL in 293 cells resulted in titers ranging from 5×10^9 to 1×10^{10} pfu/ml, 5–10 times lower than those seen with other replication-deficient adenoviral vectors produced in our laboratory. Infection of 293 cells with adCMV-FasL resulted in rapid cell death within 8–12 hr. Transcripts for Fas (CD95) were detectable by reverse-transcription (RT) PCR in 293 cells, and it is likely that these cells express Fas. In contrast, infection with pFG140, a replication-deficient type 5 adenovirus with the insert pMX2 at 3.8 m.u. (Graham, 1984), resulted in viral cytolytic effect after more than 24 hr.

COS cells infected with 10^9 pfu of adCMV-FasL at 24 hr revealed the presence of a 36- to 38-kD protein, the size of murine FasL, as detected by Western blotting (Fig. 1). Only minute amounts of proteins with the size of FasL were detected in uninfected COS cells, or cells infected with 10^9 pfu of pFG140. To determine the biological activity of recombinant FasL, we evaluated the ability of adCMV-FasL to induce apoptosis of Fas-positive target cells. COS cells infected with 10^8 pfu of adCMV-FasL induced apoptosis of 66.7 ± 2.9% of Jurkat cells after co-incubation for 6 hr, versus 5.2 ± 0.1% with

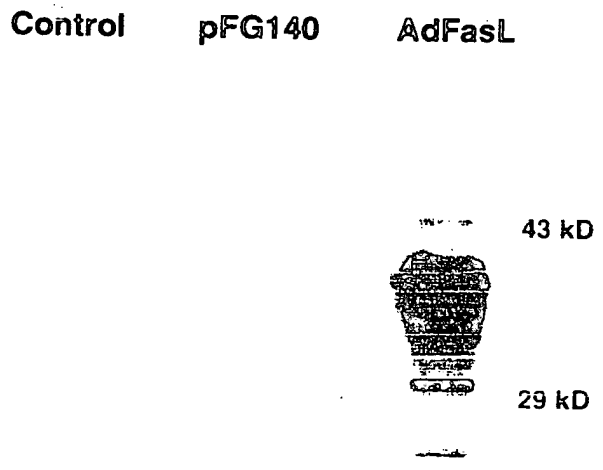


FIG. 1. Fas ligand Western blotting. COS cells infected with 10^9 pfu of adCMV-FasL express a 36 to 38 kD protein consistent with murine Fas ligand. Control uninfected and pFG140-infected cells were negative.

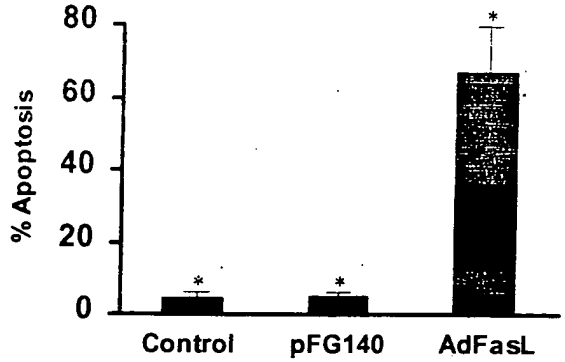


FIG. 2. Induction of apoptosis *in vitro*. A total of 66.7% of Jurkat cells co-incubated with adCMV-FasL-infected COS cells underwent apoptosis at 6 hr, versus 5.2% with pFG140 and 4.3% with uninfected COS cells ($n = 3$, $p < 0.005$). Bars indicate 95% confidence intervals.

COS cells infected with 10^8 pfu of pFG140 and 4.3 ± 0.4% using uninfected cells ($n = 3$, $p < 0.005$) (Fig. 2).

Effects of systemic administration of AdCMV-FasL

To analyze the *in vivo* effect of adCMV-FasL, rats and mice were exposed to adCMV-FasL by intravenous infection. Rats infused with 6.8×10^9 pfu adCMV-FasL died within a 72-hr period; however, control rats given a similar dose of adCMV-βgal all survived to sacrifice. DBA/2J mice given 1×10^9 pfu of adCMV-FasL also died within 24 hr; however, DBA/2J mice given the equivalent dose of adCMV-βgal or *lpr* mice (which have a mutation in Fas) given the same dose of adCMV-FasL survived. Administration of 10^9 pfu or less of adCMV-FasL to rats and 4×10^8 pfu or less to DBA/2J mice did not result in death when followed up to 10 days post administration.

At 72 hr after infection with adCMV-FasL, histopathology of surviving rat and DBA/2J mouse livers demonstrated significant hepatocellular damage with diffuse apoptosis, consisting of intense eosinophilic cytoplasmic staining and dense chromatin, findings that were not evident in *lpr* mice or animals transduced with adCMV-βgal (Plate 1). Interestingly, polymorphonuclear cells were more prominent in the livers of adCMV-FasL-infected animals. Staining of rat and DBA/2J mouse livers using the TUNEL assay to detect apoptosis revealed strongly positive staining in animals treated with adCMV-FasL, but not in those treated with adCMV-βgal (Plate 1). Hepatic apoptosis was not detected by either conventional histology or by TUNEL staining in *lpr* mice that received adCMV-FasL systemically.

RT-PCR of liver RNA revealed transcripts for FasL and adenoviral fiber protein in adCMV-FasL-infected animals, confirming successful transduction of hepatocytes with our construct. Similarly, animals infected with adCMV-βgal had transcripts detectable for *E. coli* β-Gal and adenoviral fiber protein, whereas mRNA for FasL, β-Gal, or adenoviral fiber protein was not detected in the livers of uninfected animals (data not shown).

PLATE 1. Liver histopathology and TUNEL staining. A. Rat liver 72 hr post 1×10^9 pfu intravenous adCMV-βgal demonstrating widespread X-Gal staining, and intact liver parenchyma (20×) (Hematoxylin & Eosin). B. Rat liver 72 hr post 5×10^8 pfu intravenous adCMV-FasL demonstrating significant hepatocellular destruction (20×) (Hematoxylin & Eosin). C. DBA/2J liver 24 hr post 4×10^8 pfu intravenous adCMVFasL. Widespread cell death marked by intense eosinophilic cytoplasmic staining and condensed chromatin typical of apoptosis (40×) (Hematoxylin & Eosin). TUNEL staining of rat livers 72 hr following the administration of 1×10^9 pfu of intravenous adCMV-βgal (D) or adCMV-FasL (E) (20×). Administration of adCMV-FasL intravenously results in widespread hepatic apoptosis as depicted by green immunofluorescent staining of apoptotic cells.

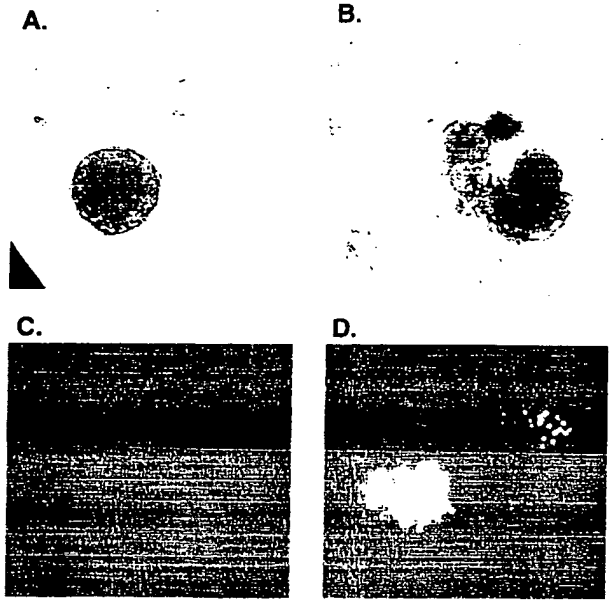


FIG. 3. AdCMV-FasL induction of pancreatic islets *in vitro*. Light microscopy of DBA/2J islets infected with pFG140 (A) or adCMV-FasL (B) (moi of 20:1) at 48 hr *in vitro*. Islets infected with adCMV-FasL are degenerating, surrounded by many free-floating cells, whereas pFG140-infected islets are [illegible]. TUNEL staining of pFG140 (C) and adCMV-FasL- (D) infected islets (moi 20:1) at 48 hr *in vitro*. AdCMV-FasL-infected islets demonstrate marked green immunofluorescence representing apoptosis.

PLATE 2. Immunohistochemistry of pancreatic islet grafts. Immunohistochemistry for murine Fas ligand on control uninfected islet isograft at 72 hr (A), adCMV-βgal-infected islet isograft at 72 hr (moi 20:1) (B), adCMV-FasL-infected islet isograft at 24 hr (moi 20:1) (C), adCMV-FasL infected islet allograft at 48 hr (moi 20:1) (D), and adCMV-FasL-infected islet isograft at 72 hr (moi 20:1) (E) (20×). Pancreatic islet grafts infected with adCMV-FasL demonstrate increasing amounts of Fas ligand expression over time as seen by yellow-brown peroxidase staining, compared with only background staining seen in uninfected and adCMV-βgal-infected islet grafts. (Islets are depicted by arrows).

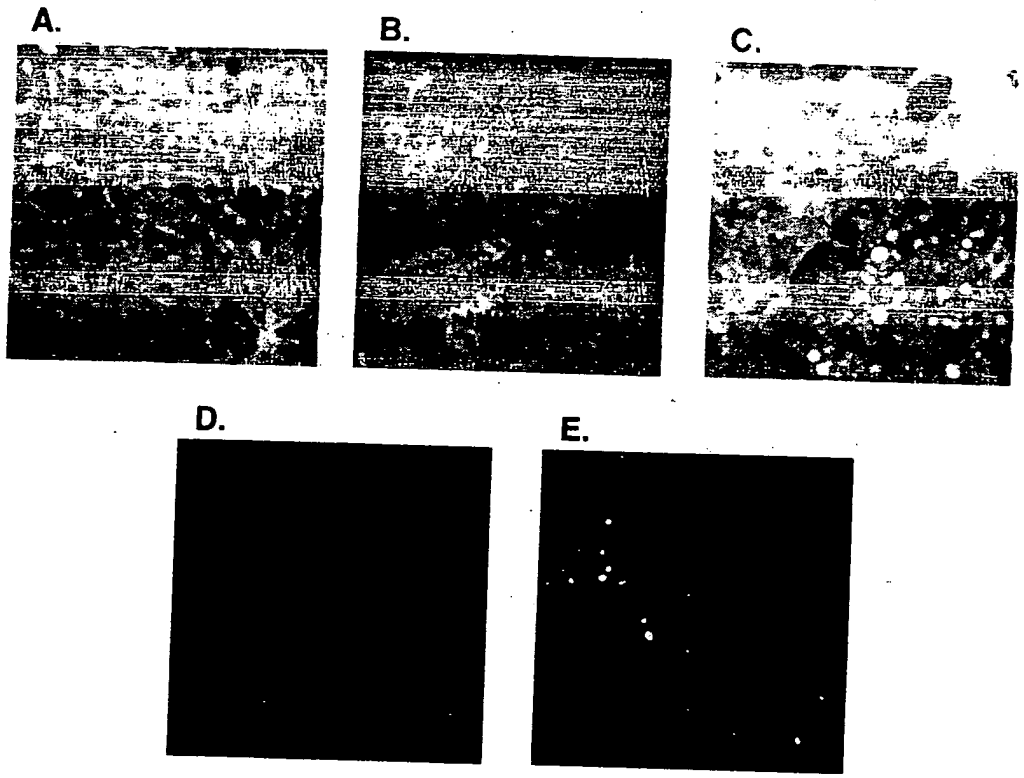
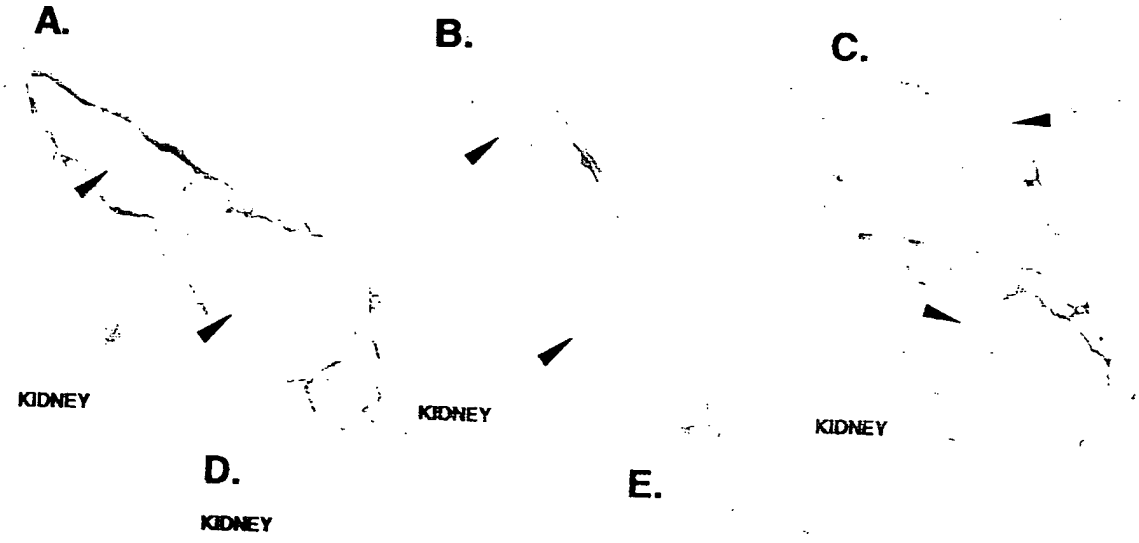


PLATE 1.



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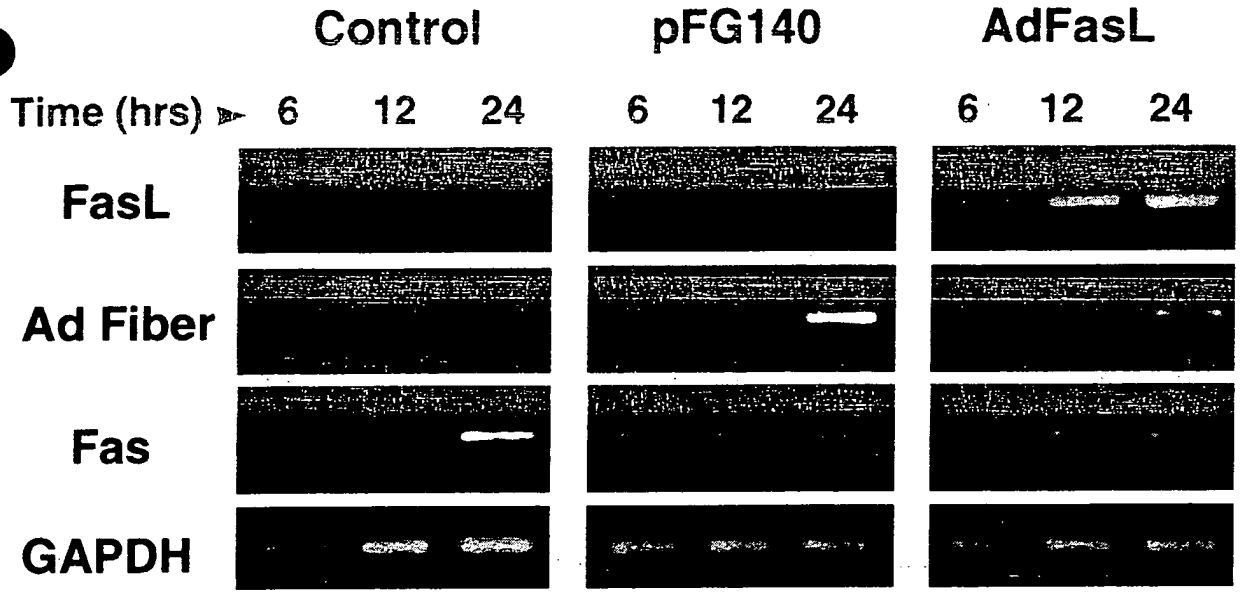

FIG. 4. Expression of Fas ligand in pancreatic islets *in vitro*. Total RNA was obtained from uninfected pancreatic islets or pancreatic islets infected with pFG140 or adCMV-FasL (moi 20:1) after incubation for 6, 12, or 24 hr. RT-PCR revealed transcripts for Fas ligand and adenoviral fiber protein increasing over time in adCMV-FasL-infected islets. In pFG140-infected islets, only mRNA for adenoviral fiber protein was detectable. Control uninfected islets had no detectable mRNA for either gene. mRNA for Fas (CD95) was detectable at all time points in control, pFG140-, and adCMV-FasL-infected islets.

Expression of FasL in AdCMV-FasL-infected pancreatic islets in vitro

Pancreatic islets infected with adCMV-FasL or pFG140 (moi 20:1) were cultured *in vitro* for 24 or 48 hr and then analyzed for the presence of apoptosis using the TUNEL assay. Morphologically, there was a striking difference between adCMV-FasL-infected islets and pFG140-infected islets (Fig. 3A,B). Whereas pFG140-infected islets appeared to be intact and round, adCMV-FasL-infected islets showed different degrees of decomposition as evidenced by the presence of many individual cells floating in the medium and the loss of the distinct round morphology of intact islets. AdCMV-FasL infection resulted in progressive islet apoptosis at 24 and 48 hr, whereas those infected with equivalent amounts of pFG140 revealed no evidence of apoptosis (Fig. 3C,D). The kinetics of Fas ligand expression in pancreatic islets following infection with adCMV-FasL was studied using RT-PCR. Pancreatic islets infected with a moi of 20:1 were first cultured *in vitro* for 6, 12, or 24 hr, followed by total RNA isolation. As expected, islets infected with adCMV-FasL had detectable transcripts for FasL and adenoviral fiber protein at all time points, whereas islets infected with pFG140 only had transcripts detectable for adenoviral fiber protein (Fig. 4). Uninfected control islets had neither FasL or adenoviral fiber protein mRNA detectable. Deoxyribonuclease-treated samples without reverse transcriptase used as controls were all negative (data not shown).

Transcripts for Fas (CD95) were detectable by RT-PCR in RNA samples obtained from adCMV-FasL-infected, pFG140-infected, and uninfected islets at all time points *in vitro* (Fig. 4). However, no Fas was detectable by immunohistochemistry performed on pancreatic islet isografts, suggesting that Fas is expressed by pancreatic islets, but at low levels.

Pancreatic islet transplantation

FasL expression by testis and cornea has been linked with tissue-specific immune-privilege (Bellgrua *et al.*, 1995; Griffith *et al.*, 1995). In addition, delayed rejection is seen when pancreatic islet allografts are co-transplanted with FasL-expressing myoblasts (Lau *et al.*, 1996). Hence, adCMV-FasL was tested as a means to prevent rejection in our murine pancreatic islet transplant model. C3H/HeJ and DBA/2J islets were infected with adCMV-FasL at a moi of 20:1 or 2:1, and transplanted under the renal capsule of streptozotocin-treated diabetic C57BL/6 or B6A/F1 recipients. All recipients of islet allografts infected with a moi of 20:1 of adCMV-FasL experienced primary nonfunction of their grafts, defined as whole blood glucose levels >200 mg/dl 72 hr post transplant ($n = 4$). In comparison, uninfected islet allografts had primary function and rejected their grafts on day 16.5 ± 1.6 ($n = 4$, $p < 0.005$). Pancreatic islet allografts infected with a moi of 2:1 experienced primary function; however, all went on to lose graft function at 8.0 ± 0.6 days ($n = 4$).

To examine whether the primary islet graft nonfunction was due to accelerated rejection or a direct effect of adCMV-FasL, DBA/2J islets were infected with adCMV-FasL or adCMV-βgal with a moi of 20:1, and transplanted under the renal capsule of syngeneic diabetic DBA/2J recipients. Syngeneic DBA/2J islet grafts lost function at 5.8 ± 0.5 days post transplant when infected with adCMV-FasL ($n = 5$), whereas DBA/2J islet isografts infected with adCMV-βgal had normal function post-transplant and prolonged survival of >50 days ($n = 5$, $p < 0.001$). Islet isografts infected with adCMV-βgal all exhibited strong X-Gal staining at all time points post-transplant.

At a moi of 20:1, histopathology of adCMV-FasL-infected islet allografts and isografts harvested at 72 hr demonstrated

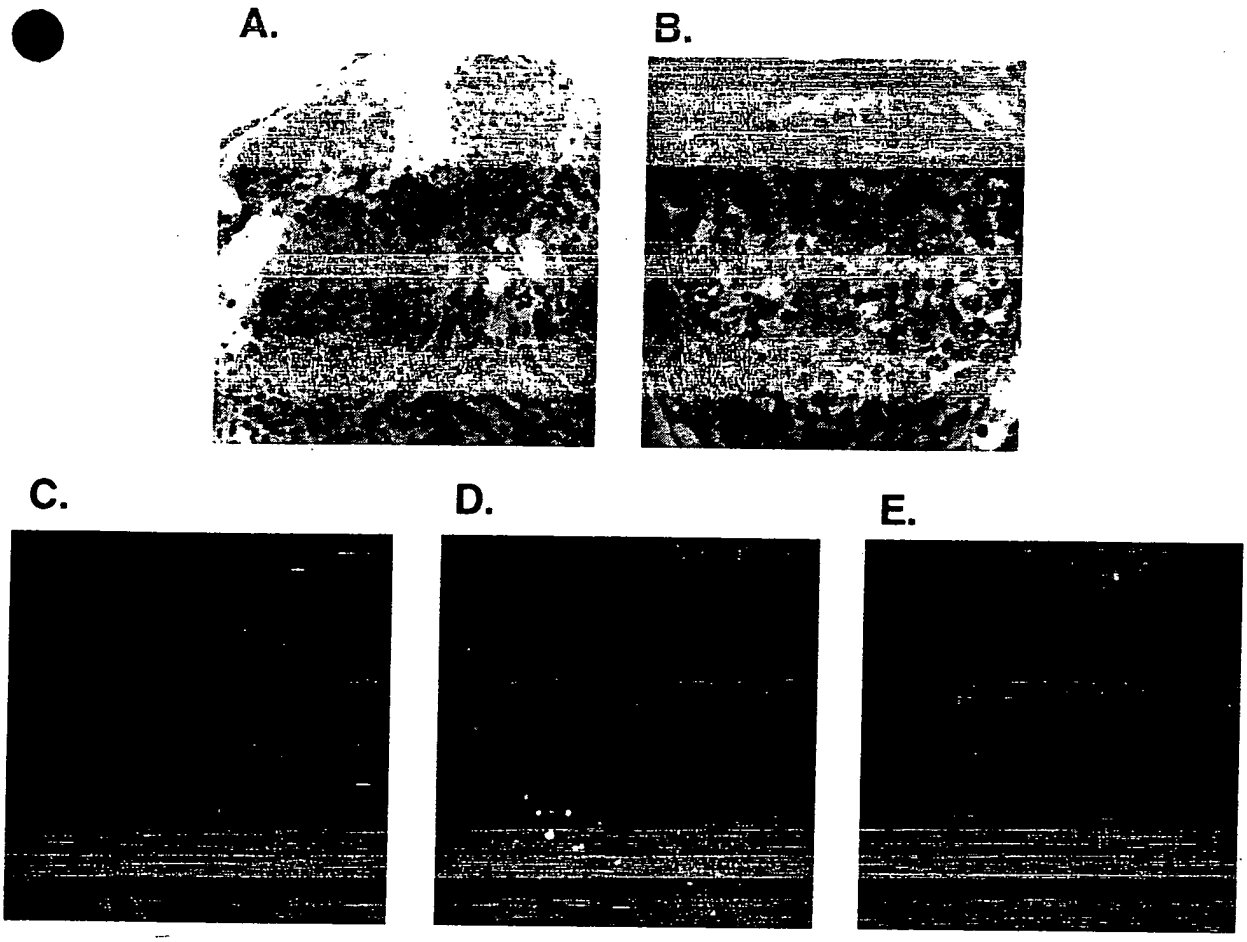

FIG. 5. Pancreatic islet graft histopathology and TUNEL staining. A. AdCMV-βgal-infected pancreatic islet isograft (moi 20:1) 72 hr post transplant. Islets are intact with minimal cellular infiltration (20×) (Hematoxylin & Eosin). B. AdCMV-FasL-infected pancreatic islet allograft (moi 20:1) 72 hr post transplant demonstrating degenerating islet surrounded by prominent neutrophilic infiltrate (40×) (Hematoxylin & Eosin). TUNEL staining of adCMV-βgal-infected islet isograft (C) (20×), adCMV-FasL-infected islet isograft (D) (20×), and adCMV-FasL-infected islet allograft (E) (10×). *Ex vivo* infection of pancreatic islet grafts with adCMV-FasL results in widespread islet apoptosis.

widespread destruction of the islets with a significant polymorphonuclear cell infiltration. In contrast, islet isografts infected with equivalent amounts of adCMV-βgal were intact, with no polymorphonuclear cells seen (Fig. 5). TUNEL staining of pancreatic islet allografts and isografts revealed widespread apoptosis that was not present in adCMV-βgal-infected islet isografts (Fig. 5). Pancreatic islets infected with a moi of 2:1 also demonstrated significant islet destruction due to apoptosis, but with less severity, likely explaining the primary function and slightly longer allograft survival.

To confirm expression of FasL *in vivo*, immunohistochemistry of pancreatic islet grafts was performed. Pancreatic islets infected with adCMV-FasL (moi of 20:1) demonstrated positive staining for FasL at 24 hr, with increased intensity at 48 and 72 hr, but not in adCMV-βgal or uninfected islets (Plate 2). Pancreatic islet isografts were also studied for the presence of FasL transcripts. FasL mRNA was detectable in islet isografts infected with adCMV-FasL at 24 and 72 hr, but not in adCMV-βgal-treated or control uninfected grafts (Fig. 6). Deoxyribonuclease-treated samples without reverse transcriptase used as controls were all negative (data not shown).

DISCUSSION

In this report, we describe the generation of a Fas-L expressing, replication-deficient adenovirus and its biological effects. Fas is expressed on most cells and tissues, although expression is most abundant in thymus, liver, heart, kidney, and ovary (Watanabe-Fukunaga *et al.*, 1992). 293 cells, which are a transformed human embryonic kidney cell line (Graham *et al.*, 1977), express Fas, and thus construction of adCMV-FasL may have been limited by cell death induced by FasL expression during the initial co-transfection with pJM17 and pAC-

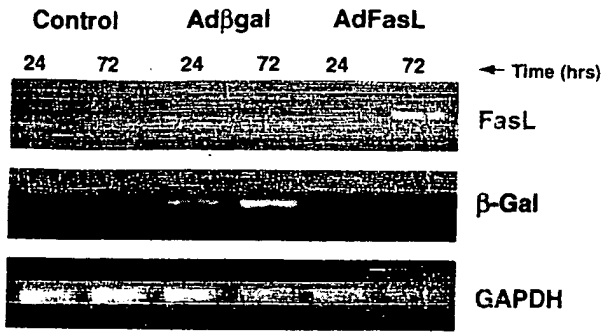

FIG. 6. Expression of Fas ligand *in vivo*. RT-PCR was performed on total RNA obtained from control, adCMV-βgal-, and adCMV-FasL- (moi 20:1) infected islet isografts 24 and 72 hr post-transplantation. mRNA for Fas ligand and β-Gal are detectable in adCMV-FasL- and adCMV-βgal-infected islets, respectively, increasing over time. Control uninfected islet isografts are negative for both Fas ligand and β-Gal transcripts.

CMV.pLpA-FasL. Despite the rapid cell death demonstrated in the 293 cells during co-transfection, a recombinant was obtained, probably arising either in cells not undergoing apoptosis, or due to viral replication occurring before apoptotic cell death. Nevertheless, the fact that the 293 cells likely undergo apoptosis upon infection with adCMV-FasL is the likely explanation for the low viral titers obtained during propagation of adCMV-FasL in this cell line.

As expected, expression of FasL in COS cells was capable of inducing apoptosis in Jurkat cells *in vitro*. The *in vivo* effects of adCMV-FasL are also not totally unexpected. It has been previously demonstrated that activation of Fas with an anti-Fas antibody results in severe liver damage by apoptosis and animal death (Ogasawara *et al.*, 1993). More recently, a vaccinia virus encoding murine FasL also caused hepatic apoptosis when administered intravenously to mice (Ehl *et al.*, 1996). In addition, expression of FasL in T lymphocytes and T cell lines results in cell death in an autocrine manner, likely via Fas (Dhein *et al.*, 1995). Thus, widespread apoptosis in rat and mouse hepatocytes induced by adCMV-FasL is consistent with the similar observation that expression of FasL leads to cell death, probably also in an autocrine manner, although cell-to-cell interaction and bystander effects resulting in cell death may also be occurring.

Expression of FasL in certain tissues such as testis or cornea results in immune-privilege (Bellgrau *et al.*, 1995; Griffith *et al.*, 1995). Lau *et al.* demonstrated that FasL expression by myoblasts in the vicinity of pancreatic islet allografts results in graft prolongation and the abrogation of rejection (Lau *et al.*, 1996). In contrast to these findings, Ehl *et al.*, using a vaccinia virus encoding murine FasL, demonstrated no evidence for susceptibility of virally activated cytotoxic T lymphocytes to FasL-mediated cell death *in vivo* (Ehl *et al.*, 1996). We demonstrate that expression of FasL directly within pancreatic islets using adCMV-FasL results in apoptosis and destruction of both syngeneic and allogeneic grafts. Although a viral effect cannot be completely ruled out, the fact that infection with adCMV-βgal did not result in islet destruction ensures that FasL expression is central to graft failure. Infection of islet allografts with a lower titer of adCMV-FasL avoided primary nonfunction due to less severe islet apoptosis, but graft failure ultimately was not prevented.

It is unlikely that the cell death demonstrated in our experiments is related solely to viral infection for several reasons. First and foremost is the observation that *lpr* mice do not experience any adverse effects after systemic administration of identical amounts of adCMV-FasL. Second, infection of pancreatic islets or systemic administration using equivalent titers adCMV-βgal does not result in similar islet or hepatic apoptosis, or animal death. Third, islets infected with adCMV-FasL undergo apoptosis in culture. The viral nature of adCMV-FasL, however, may be more lethal than anti-Fas antibodies or nonviral expression of FasL. Fas expression is increased on cells infected with other viruses such as human immunodeficiency virus (HIV) (Katsikis *et al.*, 1995) and Epstein–Barr virus (EBV) (Falk *et al.*, 1992), and a number of cytokines including interferon-γ (IFN-γ; Watanabe-Fukunaga *et al.*, 1992) can increase Fas expression. Stassi *et al.* demonstrated that interleukin-1β (IL-1β) can induce Fas expression on purified human pancreatic β cells (Stassi *et al.*, 1995), and, therefore, it is possible that infection with replication-deficient adenoviruses also upregulates expression of Fas. In addition, infection of cells and tissues with replication-deficient adenoviruses may make them more susceptible to apoptosis because the vectors lack the anti-apoptotic E1B region (Boyd *et al.*, 1994). In our experiments, Fas mRNA was detectable within pancreatic islets *in vitro* with RT-PCR, but was not detectable within islet grafts by immunohistochemistry, suggesting that Fas is expressed in islets, but at low levels.

The induction of apoptosis with adCMV-FasL in cells and tissues of three different species (mouse, rat, and human) in our experiments confirms the biological relevance of FasL homology among animal species. Aside from apoptosis, the effect of expressing FasL in tissues that do not normally express the protein has not been well studied. Yagita *et al.* suggested that BHK fibroblasts or lymphoma cell lines expressing human FasL were chemotactic to granulocytes when transplanted into xenogeneic nude mice or syngeneic animals (Yagita *et al.*, 1996). The observation of neutrophilic infiltrates in our systemically treated animals and in the adCMV-FasL-infected islet grafts is consistent with those results. Granulocytes constitutively express Fas and readily undergo apoptosis, a process that may be critical for their removal from inflammatory sites (Liles *et al.*, 1996). We have no evidence whether the granulocytes infiltrating the islet grafts undergo apoptosis when in contact with FasL-expressing islet cells. However, because only a small percentage of infiltrating granulocytes would get in contact with the FasL-expressing islets, only those cells would undergo apoptosis. The role of the polymorphonuclear cells within our islet grafts is not known, but may be a nonspecific response to surrounding cell death. Apoptosis of the islets might either trigger an inflammatory response or lead to release of chemokines that attract granulocytes.

The Fas/FasL pathway has also been implicated in other disease systems including certain cancers such as lymphomas, malignant gliomas, and cancers with p53 mutations and autoimmune disorders such as systemic lupus erythematosis (Thompson, 1995). Despite the disappointing results in our transplant model, adCMV-FasL is a potentially useful tool to study further the effects of FasL expression in nonlymphoid tis-

sues both in terms of Fas/FasL biology and the potential for therapy in situations where localized cell death might be beneficial.

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ELSEVIER

Rejection of Fas Ligand-Expressing Grafts

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FAS is a type I integral membrane protein which transduces an apoptotic death signal.[1] Fas is expressed in hematopoietic cells and various tissues including the liver, lung, intestine, and skin. On the other hand, Fas ligand (FasL) is a type II integral membrane protein that can induce apoptosis in Fas-bearing cells.[1] Fas/FasL interaction has been shown to regulate immune responses and induce some tissue damage.[1,2] Recent reports suggested that constitutively expressed FasL in the testis and eye contributes to preventing graft rejection and tissue destruction.[3,4] However, we recently found a contradictory phenomenon that subcutaneously transplanted FasL-expressing xenogeneic cells were acutely rejected.[5] In this study, we show a bystander rejection induced by FasL-expressing cells.

MATERIALS AND METHODS

Animals

Six week-old female BALB/c nude and DBA/2 mice were purchased from Charles River (Atsugi, Japan).

Cells

Fas-negative baby hamster kidney (BHK) cells were obtained from American Type Culture Collection. Fas-negative mouse T lymphoma L5178Y derived from DBA/2 mouse was kindly provided by Dr S. Yonehara (Kyoto University, Kyoto, Japan). Human and mouse FasL (hFasL and mFasL) cDNA were transfected into BHK and L5178Y as described previously.[6] These cells were maintained in RPMI 1640 (Nissui, Co Ltd, Tokyo) supplemented with 10% FCS, 2 mM of glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin.

In vivo evaluation of tumor growth

Mice (five per group) were injected subcutaneously with 2×10^6 tumor cells in 200 μL PBS and tumor growth was assessed twice per week. Some mice inoculated with hFasL transfectants were intraperitoneally administrated with 100 μg of a neutralizing anti-hFasL mAb (NOK-1) three times per week. For estimating the bystander rejection, DBA/2 or BALB/c nude mice (five per group) were subcutaneously inoculated with parental cells (2×10^6) and the same number of hFasL transfectants at the same site or a distant site. Tumor growth of parental cells was examined twice per week.

RESULTS

We first established human and mouse FasL transfectants from Fas-negative BHK and L5178Y ($H\text{-}2^d$) mouse lymphoma cell line (hFasL/BHK, mFasL/BHK, hFasL/L5178Y, and mFasL/L5178Y, respectively). The FasL transfectants exhibited high Fas-dependent cytotoxic activities. Growth rates of the FasL-transfected and parental cells in vitro were almost equal. When these cells were subcutaneously inoculated into syngeneic DBA/2 or BALB/c nude mice, the parental cells grew well but their FasL transfectants were acutely rejected as we previously reported using BHK and its FasL transfectants.[5] Administration of NOK-1 reversed the rejection of hFasL transfectants, indicating that it was induced by FasL.

Because the rejection was observed in nude mice, it is apparently independent of cytotoxic T cells. The mice transplanted with FasL transfectants exhibited no abnormality and survived as long as naive mice. While the parental cells grew when transplanted apart from the FasL transfectants, they were completely rejected when transplanted together with the FasL transfectants (Fig 1A–C). The growth of L5178Y cells transplanted apart from hFasL/L5178Y cells was slowed in DBA/2 mice (Fig 1C), suggesting that some T cell-dependent tumor inhibition was operative.

DISCUSSION

In this study, we demonstrated a FasL-mediated graft rejection. The mechanism underlying the FasL-mediated rejection has been investigated using some FasL-transfected tumor cells;[7] briefly, the rejection occurred not only subcutaneously but also intraperitoneally in syngeneic mice. Because the rejection was abrogated by depletion of granulocytes and massive neutrophil infiltration was observed at the rejection site, it seemed to be induced mainly by neutrophils.

In our study, the FasL transfectants were more susceptible to neutrophil-mediated killing than the parental cells.[7] This suggests that FasL activates some cytotoxic machinery in neutrophils. The bystander rejection of the parental cells might be induced by a local effect of neutrophils which was activated by composite inoculation of the FasL transfec-

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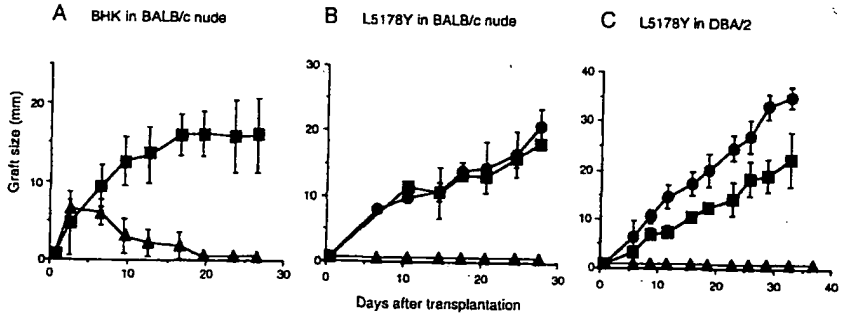

Fig 1. Bystander rejection of parental BHK and L5178Y cells in BALB/c nude or DBA/2 mice. The 2 × 10^6 cells of parental BHK (A) and L5178Y (B, C) were injected simultaneously with the same number of hFasL/BHK (A) or hFasL/L5178Y (B, C) into BALB/c nude (A, B) or DBA/2 (C) mice. The cells were injected at the same site (▲), or distant sites (■), and tumor growth was measured at where the parental cells were injected. In B and C, the growth of parental L5178Y cells alone (●) were also included. Data represent mean ± SE of five animals.

tants. The parental cells grew when transplanted at a distant site from the FasL transfectants, indicating that the effect of FasL is locally limited.

Recently, Bellgrau et al[3] reported that testicular grafts derived from wild-type mice that express FasL survived indefinitely when transplanted under the kidney capsule of allogeneic recipients. However, our present results are contradictory to their observation. To investigate what led to the discrepancy, we transplanted the FasL transfectants under the kidney capsule and recently found that the FasL transfectants were maintained under the kidney capsule (manuscript in preparation). This result indicates that the site of transplantation substantially affects the graft acceptance even if the graft expresses FasL.

It is expected that the manipulation of tissues to express FasL may be feasible for preventing graft rejection. The kidney capsule would be an appropriate site for establishing the FasL-mediated immune privilege, while other sites such as subcutaneous or intraperitoneal region cannot be suitable. Indeed, the strategy facilitating the graft acceptance by local FasL expression is attractive and important in light of clinical transplantation. To succeed its application to humans, it seems necessary to further verify in vivo function of the Fas/FasL system.

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NOTES

Application of a Fas Ligand Encoding a Recombinant Adenovirus Vector for Prolongation of Transgene Expression

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An adenovirus vector encoding murine Fas ligand (mFasL) under an inducible control was derived. In vivo ectopic expression of mFasL in murine livers induced an inflammatory cellular infiltration. Furthermore, ectopic expression of mFasL by myocytes did not allow prolonged vector-mediated transgene expression. Thus, ectopic expression of functional mFasL in vector-transduced cells does not appear to confer, by itself, an immunoprivileged site sufficient to mitigate adenovirus vector immunogenicity.

Recombinant adenovirus vectors have found broad utility for a variety of gene therapy applications (9, 57). This fact derives principally from their ability to accomplish efficient in vivo gene transfer in a variety of organ contexts. Despite this property, successful use of these agents for gene therapy purposes has been significantly limited to date, largely because an invariable consequence of in situ cellular transduction by adenovirus vectors at distinct parenchymal sites has been shown to be a significant host immunological response against transduced cells (52, 56, 60). A number of specific immune effector mechanisms, together with nonspecific defense mechanisms, are called into play to eliminate an infecting virus (57). This process has been associated with attenuation of expression of the transferred therapeutic gene based, at least in part, on loss of the vector-transduced cells (12, 61, 63). Since the cells infected with recombinant adenoviruses are usually rapidly eliminated, it is likely that the host immune system plays a major role in preventing sustained expression of the foreign genes. Innate and adaptive immune response-related clearance of adenovirus vectors in vivo has been described (59). The importance of the immune response against adenovirus vectors was first suggested by reports of long-term recombinant gene expression and less inflammatory reaction after a single adenovirus administration to neonatal animals, which have an immature immune system (64). Similar results were obtained in mice with severe immunodeficiency and in nude mice (6). Subsequently, several groups have demonstrated that infection of an immunocompetent host with recombinant adenoviruses elicits a $CD8^+$ cytotoxic T-cell (CTL) response that eliminates virus-infected cells within 28 days of infection (12, 61, 62). Thus, strategies for prolonging the expression of therapeutic genes delivered by adenovirus, even in the context of diseases in which transient effects may be sought, such as cancer, are essential requirements for achieving clinical utility.

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Based on a growing understanding of the immunological phenomena underlying this process, a variety of distinct strategies have been proposed to attenuate vector immunogenicity (31). To maximize the therapeutic potential of adenovirus vectors, various treatments administered at the time of vector delivery, aimed at modifying the host immune response, are being developed. In this regard, it has been postulated that the major stimulus for host immune responses is the expression of endogenous viral genes by transduced cells (63). Consequently, strategies to reduce endogenous viral gene expression through additional deletions in several gene regions of recombinant adenovirus vectors have been developed (16, 55). Direct strategies to abrogate presentation of viral antigens by antigen-presenting cells to the immune system have also been studied. Methods used have included cytotoxic drugs, antilymphocyte agents, cyclosporine, FK506, and deoxyspergualin (21, 28, 53, 58). In addition, interruption of the specific interaction between major histocompatibility complex class I molecules in antigen-primed cells and helper T lymphocytes has been proposed. Interventions explored in this context have included the use of agents that block costimulatory signals, such as CTLA4Ig and anti-CD40 ligand (19, 25). Thus, considerable efforts have been directed at mitigating host response to the vector-transduced cells as a means to prolong transgene expression for gene therapy purposes.

From a conceptual standpoint, these strategies seek to render vector-transduced cells into immunologically privileged sites to avoid their recognition by the host immune system. In this regard, host mechanisms for establishing such immune privilege have been recognized to occur in selected endogenous physiological contexts. Immune privilege in sites like the eye, testis, and brain allows foreign agents and tissues to persist in those locations, and this phenomenon has long held the promise of solving the problems of autoimmunity, graft rejection, and potentially vector immunogenicity (14, 18, 29, 39, 45, 54). One clinical example of the function of an immunologically privileged site is the success of human corneal transplants, where a very high percentage of transplants engraft without tissue matching or immunosuppressive therapy. Stuart et al.

recently demonstrated that Fas ligand (FasL) expression on the cornea is a major factor in corneal allograft survival; thus, they provide an explanation for one of the most successful tissue transplants performed in humans (50). FasL is a member of the tumor necrosis factor family and induces apoptosis in cells that express Fas (7, 20, 43, 51, 54). FasL is expressed by activated CTLs as well as NK cells and works as a death induction factor (33, 35). It has been proposed that when activated inflammatory cells enter the eye or testis, they are immediately killed through the Fas-FasL pathway (17, 34). In this regard, Lau et al. showed that syngeneic myoblasts expressing murine FasL (mFasL) protected allogenic pancreatic islets concomitantly transplanted under the kidney capsule (27). Furthermore, the protective effect of FasL was also observed when testis-derived Sertoli cells survived and provided local immunosuppression for xenografts in rat brains (47). Recently, Xu et al. have shown an evasion of the immune CTL response by induction of FasL expression on simian immunodeficiency virus-infected cells (60). Malignant melanoma and hepatocellular carcinomas have been found to express FasL, suggesting that these tumor cells can evade the immune attack through their expression of FasL (20, 49). These remarkable observations have led to the hypothesis that ectopic FasL expression may have the potential to render a site impervious to the consequences of immune recognition. It thus seemed reasonable to use such an approach to achieve immune protection of vector-transduced cells in a gene therapy context. We hypothesized that such immune protection of cells transduced by adenovirus might be induced via methods directed at local augmentation of expression of the FasL molecule. Furthermore, this attenuation of the host immune response might thus allow prolongation of the transgene expression that derives from transduction with recombinant adenovirus vectors.

The initial step in this endeavor was the construction of a replication-incompetent, recombinant adenovirus vector expressing mFasL. A first consideration was the fact that coexpression of Fas and FasL in the same cellular context results in an autocrine loop that induces apoptosis (26). Thus, a Fas-positive phenotype in available packaging cells (293 cells) would potentially undermine efforts to derive a FasL-expressing vector. In this regard, at 10 h after infection with 5 PFU per cell of AdCMVLuc (irrelevant virus), AdLoxpFasL alone, or AdLoxpFasL plus AdCANCre, both of which express the inducible mFasL, we analyzed apoptosis in 293 cells. Cells were washed with phosphate-buffered saline and resuspended at 10^6 cells/ml. Early detection of apoptosis was performed with an ApoAlert Annexin V Fluos staining kit as instructed by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). Detection of apoptosis is based in changes occurring on the cell surface during early stages of apoptosis; specifically, translocation of phosphatidylserine from the interior side of the plasma membrane to the outer leaflet is detected. This is the basis for the high-affinity binding of annexin V to phosphatidylserine. For this analysis, 293 cells were incubated with annexin V-biotin in a HEPES buffer for 15 min at room temperature. Fluorescence-activated cell sorting analysis was performed in 10^4 events in a pool of cells from quadruplicate experiments, and data were expressed as a percentage of apoptotic cells (Fig. 1). The percentage of cells within each region was calculated by using CellFIT version 1.0 (Becton Dickinson).

We thus conceptualized a strategy to express FasL in an inducible context, based on an application of the Cre/Loxp system to the recombinant adenovirus context (5). Figure 2A depicts the structure of our designed vector, whereby FasL is functionally separated from the modified chicken β-actin promoter with the cytomegalovirus immediate-early enhancer

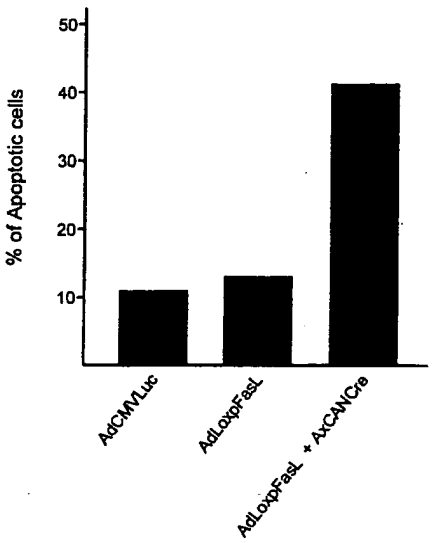

FIG. 1. Apoptosis analysis of 293 cells expressing mFasL, performed as described in the text.

(CAG) (37) by a Loxp-flanked stuffer segment. The encoded Cre recombinase protein would be predicted to excise the stuffer and allow functional reconstitution of the expression cassette at the deleted E1 site of the recombinant adenovirus vector. Of note, Kanegae et al. (22) and Anton and Graham (5) have both described the utility of such a Loxp-based inducible system in the context of the adenovirus genome. Thus, this strategy based on inducible gene expression offered the means to construct such a vector expressing FasL, regardless of the expression of Fas by the viral vector packaging cell line.

The recombinant adenovirus AdLoxpFasL was constructed by the two-plasmid homologous recombination method of Parks et al. (40). To generate a plasmid expressing mFasL incorporating the Cre/Loxp system, a β-actin promoter-driven neomycin resistance (Neo^r) gene flanked by two Loxp sites was subcloned as a *Hin*dIII-*Sal*II fragment from plasmid pCAN-LNLW (provided by I. Saito, Tokyo, Japan) into the multiple cloning site of the adenovirus shuttle plasmid pΔESP1B (Microbix, Inc., Ontario, Canada). The resultant plasmid, pΔEloxP, sequentially contains 0.5 map units of sequence from the left end of the adenovirus type 5 genome, the β-actin promoter, the first Loxp site, the Neo^r gene, a second directional repeat Loxp site, and a unique *Swa*I site, followed by simian virus 40 poly(A) signal sequences and finally map units 9 through 16 of the adenovirus genome. Full-length mFasL was excised from the plasmid pcDNA3-mFasL with *Bam*HI and *Xho*I, blunt ended with Klenow fragment, and then subcloned into the *Swa*I site of pΔEloxP. Restriction endonuclease digestion and direct sequence analysis confirmed the orientation and sequence of the inserted mFasL. The resultant plasmid, pΔE1loxPF, was then cotransfected into the adenovirus packaging cell line 293 together with the adenovirus packaging plasmid pJM17 (Microbix), by using Lipofectin (BRL, Gaithersburg, Md.) as previously described (44). After cotransfection, cells were overlaid with Dulbecco's modified Eagle's medium-F12 (Mediatech/Cellgro) supplemented with 2.5% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, Utah) and 0.65% Noble agar (Difco, Detroit, Mich.). Plaques were picked approximately 10 days posttransfection and carried through three additional isolation steps. The identity of the resultant adeno-

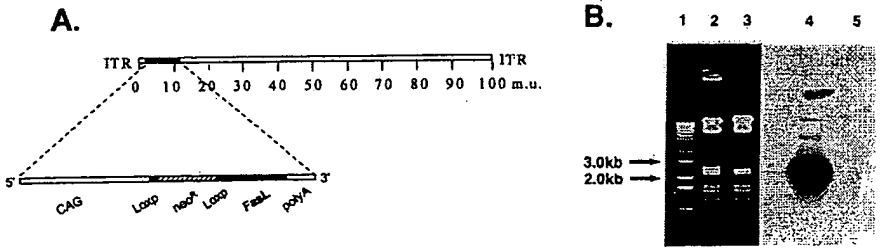

FIG. 2. Construction of a recombinant adenovirus vector encoding mFasL. (A) Map of the recombinant adenovirus vector. An expression cassette is inserted into the deleted E1A region. This cassette allows inducible expression of mFasL from the CAG promoter after excising of the stuffer Neo^r gene flanked by the Loxp sites. ITR, adenovirus inverted terminal repeats. Map units (m.u.) 0 to 100 are indicated. (B) Confirmation of identity of the adenovirus vector encoding mFasL, AdLoxpFasL. Adenovirus genome DNA was subjected to restriction endonuclease digestion with *Xho*I and analyzed by gel electrophoresis and Southern blotting. For Southern blotting, a ^{32}P-labeled mFasL probe was used. Lane 1, 1-kb DNA ladder; lane 2, *Xho*I digestion of AdLoxpFasL; lane 3, *Xho*I digestion of E1-deleted adenovirus vector lacking the mFasL gene; lane 4, Southern blot analysis as for lane 2; lane 5, Southern blot analysis as for lane 3.

virus vector, AdLoxpFasL, was confirmed by restriction endonuclease digestion with *Xho*I and Southern blot analysis using standard procedures (Fig. 2B).

We next sought to confirm the expression of mFasL from the derived adenovirus vector AdLoxpFasL. Such analysis was required to confirm the relative absence of expression from the noninduced vector configuration, as well as the mFasL expression after induction with Cre recombinase in cells transduced with the inducible vector. For this analysis, murine B6 *lpr/lpr* (*lpr* stands for lymphoproliferation) macrophages were used. These cells derive from a transgenic mouse deficient in Fas expression and thus permit FasL expression without the potential consequences of induction of apoptosis by the interaction between Fas and FasL (3, 36). Cells were grown in RPMI 1640 medium supplemented with 10% FBS in a humidified 5% CO_2 atmosphere and seeded at 10^5 cells in 60-mm^2 plates. After overnight culture, cells were coinfected with AdLoxp FasL and AxCANCre, a Cre-expressing recombinant adenovirus vector, for induction of mFasL expression in effector cells (22). As a control, cells were also coinfected with AdLoxpFasL plus a non-Cre-expressing recombinant adenovirus, AdCMV LacZ. A multiplicity of infection of 5 PFU/cell was used for each vector. Infections were allowed to proceed for 1 h in culture medium containing 2% FBS, followed by incubation for 24 h in RPMI 1640 supplemented with 10% FBS. Uninfected controls, not exposed to viral vectors, were maintained and processed in the same manner. Total RNA extraction and Northern blotting were then performed by techniques described elsewhere (65). Probes for this analysis included a 960-bp fragment of the mFasL cDNA amplified by PCR or a murine β-actin cDNA. In this analysis, the addition of the adenovirus expressing Cre recombinase resulted in a marked induction of mFasL expression in the target cells. Specifically, a readily detectable band corresponding to the full-length mFasL cDNA could be noted when RNA from the experimental group including both AxCANCre and AdLoxpFasL was analyzed. A band corresponding to the induced expression of the mFasL cDNA was not noted in other groups. Interestingly, a band of higher molecular weight was detected in the group including the AdCMVLacZ and AdLoxpFasL vectors, in relatively reduced amounts. The size of the band, as well as its context, suggests that a low level of spontaneous expression of mFasL occurred with these vectors. Levels of mFasL, however, were dramatically less than those noted when AxCANCre and AdLoxpFasL were administered. Analysis of the β-actin transcripts showed comparable levels in each group. Thus, the AdLoxpFasL vector is capable of expressing high levels of mFasL in target cells under the control of Cre recombinase (Fig. 3).

We next sought to confirm the functional activity of the mFasL encoded in the vector AdLoxpFasL. For this analysis, we employed a ^{51}Cr release assay in which a FasL-sensitive cell line (A20) was used as the target cell. The A20 cells were labeled with [^{51}Cr]sodium chromate (50 μCi/10^5 cells; Amersham, Arlington Heights, Ill.) for 1 h at 37°C. After extensive washing, the labeled A20 cells were then added to various murine cell lines, including B6 *lpr/lpr* macrophages, NIT-1 insulinoma cells, L3 microglioma cells, and F10 astrocytoma cells, at various effector-to-target (E/T) ratios. After 6 h of incubation, the specific release of the radioactive marker was determined by gamma scintigraphy as previously described (66). These cells had been preinfected with AdCMV*LacZ* and AdLoxpFasL or with AxCANCre and AdLoxpFasL at 5 PFU/cell. The spontaneous release of ^{51}Cr was determined by incubating the ^{51}Cr-labeled A20 with medium alone, whereas the maximum release was determined by adding sodium dodecyl sulfate solution (SDS) to a final concentration of 0.05%. The percentage of specific release was calculated as follows: % specific lysis = [(experimental ^{51}Cr release) − (spontaneous ^{51}Cr release)]/[(maximum ^{51}Cr release) − (spontaneous ^{51}Cr

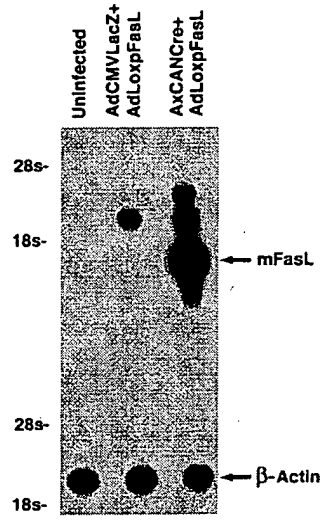

FIG. 3. Analysis of gene expression characteristic of AdLoxpFasL by Northern blot analysis. Murine B6 *lpr/lpr* mouse macrophages were infected with either AdLoxpFasL plus AdCMVLacZ (lane 2) or AdLoxpFasL plus AxCANCre (lane 3). Lane 1 is an uninfected control. Twenty-four hours postinfection, total RNA was isolated and probed with mFasL and β-actin cDNAs.

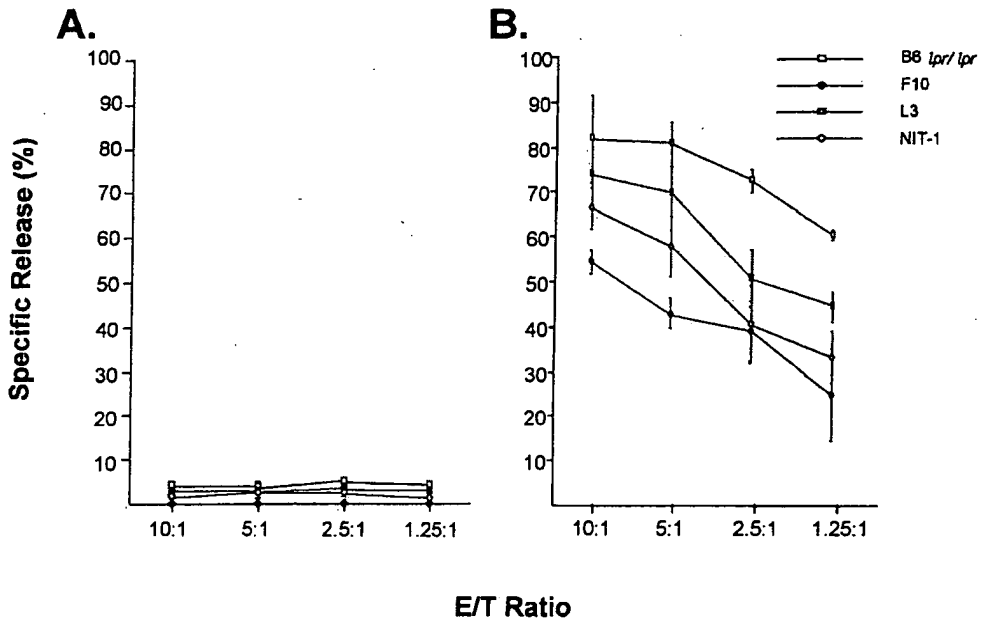

FIG. 4. Characterization of the function of ectopic expression of mFasL in macrophages. The *lpr/lpr* macrophages were infected with either AdLoxpFasL plus AdCMVLacZ (A) or AdLoxpFasL plus AxCANCre (B) and mixed with ^{51}Cr-labeled A20 cells at the indicated E/T ratios; after 6 h of incubation, the specific release of radioactive marker was determinated.

release)]. Infection with AdCMV*LacZ* and AdLoxpFasL did not induce or enhance killing in any of the tested cell lines (Fig. 4A). In marked contrast, an increase in cell killing, as manifested by ^{51}Cr release, was noted for all infected cell lines with an increasing E/T ratio (Fig. 4B) when AxCANCre and AdLoxpFasL were used. This finding is consistent with the concept that the adenovirus vector effectively mediated ectopic mFasL expression. Furthermore, it was demonstrated that the induced mFasL expression rendered target cells sensitive to killing mediated by Fas. Thus, the AdLoxpFasL vector is capable of expressing physiologically relevant amounts of functional mFasL after induction by adenovirus-mediated expression of Cre recombinase.

Whereas this experiment confirmed that mFasL augmentation could enhance cell killing via physiologic pathways of cellular interaction, we also sought to demonstrate the consequences of Fas and FasL coexpression within the same target cell. Such a finding of an autocrine pathway of induction of apoptosis would validate our strategy for construction of the adenovirus vector expressing FasL in an inducible manner. Furthermore, such a finding would have consequences for the manner whereby ectopic FasL expression would be achieved for applications to attenuate vector immunogenicity. To validate this concept, we induced expression of mFasL in HeLa and 293 cells, which already express Fas (30). For this analysis, 10^5 HeLa cells were plated in six-well tissue culture plates and cultured in Dulbecco's modified Eagle's medium-F12 supplemented with 10% FBS at 37°C and 5% CO_2 atmosphere for 24 h. The cells were then infected with the various combinations of adenovirus vectors, as described above, at 10 PFU/cell and washed extensively 1 h postinfection. At 24 h postinfection, the cells were stained with trypan blue, and triplicates were counted to determine the number of viable cells. In this analysis, induced expression of mFasL in HeLa cells resulted in a dramatic decrement in viable cell numbers (Fig. 5). This result was not seen with noninduced AdLoxpFasL. Thus, the expression of mFasL, in the context of a target cell expressing Fas, can induce an autocrine suicide event. This finding thus rationalizes the use of adenovirus-mediated ectopic expression of mFasL exclusively in tissue contexts in which Fas is not expressed.

As an additional test of the biologic effect of expression of mFasL at ectopic sites, we used AdLoxpFasL to achieve in situ expression of FasL in the liver. For this experiment, adult female C57BL/6 and B6 *lpr/lpr* mice were injected via the tail vein with adenovirus vector constructs. This means of delivery is known to achieve principally hepatocyte transduction (60). Animals were challenged with both AdCMVLacZ and AdLoxpFasL, or with AxCANCre and AdLoxpFasL, at 5×10^9 PFU per animal. Twenty-four hours after injection, livers were har-

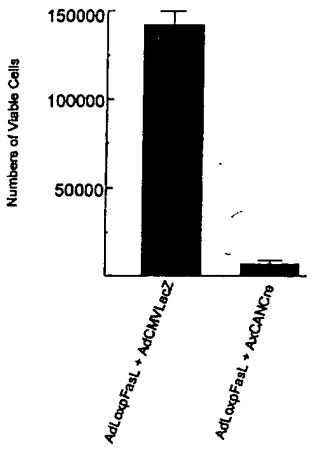

FIG. 5. Induction of autocrine suicide event by Fas-expressing target cells by AdLoxpFasL. HeLa cells were infected with AdLoxpFasL plus AdCMVLacZ or with AdLoxpFasL plus AxCANCre. After 24 h, cells were stained with trypan blue and analyzed in triplicate to determine the number of viable cells.

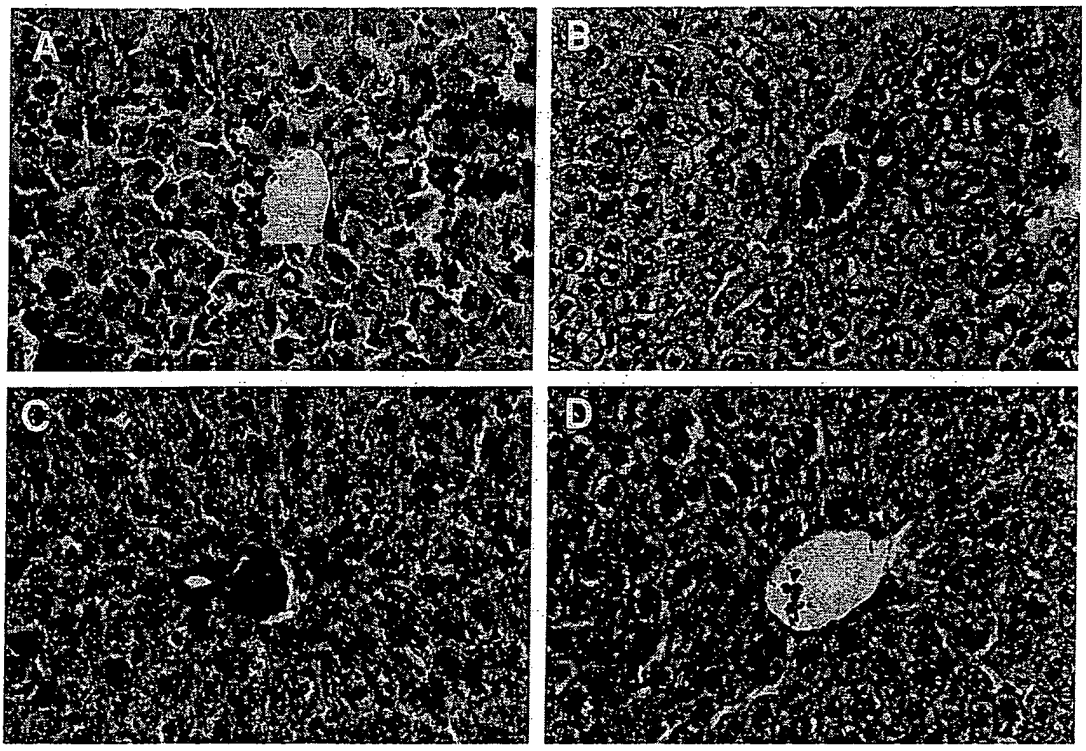

FIG. 6. Effect of ectopic expression of mFasL expression in the liver. Adult female C57BL/6 mice were injected intravenously with AdLoxpFasL plus AxCANCre (A), AdCMVLacZ (B), AdLoxpFasL plus AdCMVLacZ (C), or phosphate-buffered saline alone (D). Livers were harvested at 24 h postinjection, and sections were prepared for histological analysis staining with hematoxylin and eosin. Magnification, ×320.

vested and analyzed by immunohistochemistry to study the hepatic parenchyma. For the B6 *lpr/lpr* mice, both combinations of delivered viruses did not elicit any changes in histopathology compared to livers from control uninfected mice (data not shown). In contrast, in the group of C57BL/6 mice, which received the AxCANCre and AdLoxpFasL vectors, hepatocytes demonstrated pyknotic nuclei, as well as a significant influx of inflammatory cells (granulocytes and lymphocytes) which were distributed throughout the hepatic parenchyma. This infiltrate was not seen either in the group that received AdCMVLacZ and AdLoxpFasL or in the control group that received no virus (Fig. 6). In further analysis, detection of apoptosis in hepatic parenchyma was performed. To this end, in situ detection of apoptotic cells with Hoechst 33258 (1 μg/ml) showed that a proportion of cells underwent apoptotic changes (10, 15). In this regard, nuclei showed condensed chromatin under the fluorescence microscope (UV filter) in the treated animals with the AxCANCre and AdLoxpFasL vectors but not in the control groups (Fig. 7). The fact that this inflammatory phenomenon and apoptosis were noted only in the group in which expression of mFasL was activated by Cre recombinase suggests that it was the expression of mFasL per se which induced these alterations.

To explore the potential of mFasL to mitigate vector immunogenicity, it was necessary to use a target tissue not characterized by the expression of Fas. In this regard, myocytes are known to lack expression of Fas (46). Furthermore, there is a significant amount of data characterizing the temporal pattern of adenovirus vector-mediated transgene expression in the muscle (2, 41). Specifically, it has been shown that direct administration of adenovirus vectors by intramuscular (i.m.) injection can achieve infection of a significant number of mature myofibers (1, 42). Of note, a well-characterized host immune response is induced after i.m. delivery of adenovirus vectors, including a CTL-mediated eradication of transduced cells (62). We thus sought to mitigate this process by ectopic expression of mFasL in vector-modified myocytes. For this experiment, adult female BALB/c mice were injected i.m. via the intraglossal route to achieve transduction of mature myofibers. Groups of animals received no vector, AdLoxpFasL plus AdCMVTK plus AdCMVLuc, or AdLoxpFasL plus AxCANCre plus Ad CMVLuc (10^9 PFU of each virus per animal). The combination of viruses in the second group included an adenovirus encoding the luciferase reporter gene, AdCMVLuc, to allow measurement of transgene expression, plus AdLoxpFasL with an irrelevant control adenovirus, AdCMVTK, which would not be predicted to induce mFasL expression. The combination of viruses in the third group contained the virus expressing the reporter gene, and also contained AdLoxpFasL plus AxCAN Cre, to achieve induction of mFasL expression. Thus, this experiment would allow direct comparison of the pattern of transgene expression mediated by the adenovirus vector in the presence or absence of mFasL coexpression.

In this experiment, all uninfected control groups demonstrated an absence of luciferase expression in harvested myofibers, as expected. For the group without induced mFasL expression, an initial high level of gene expression which was readily detectable by day 7 postinfection was achieved. These levels of gene transfer underwent attenuation in a time-dependent manner such that by day 50 postinfection, the magnitudes of luciferase gene expression were nearly 4 orders of magnitude less than those observed at day 7 (Fig. 8). This pattern of nonpersistence of transgene expression is analogous to that described by other authors and reflects the consequences of

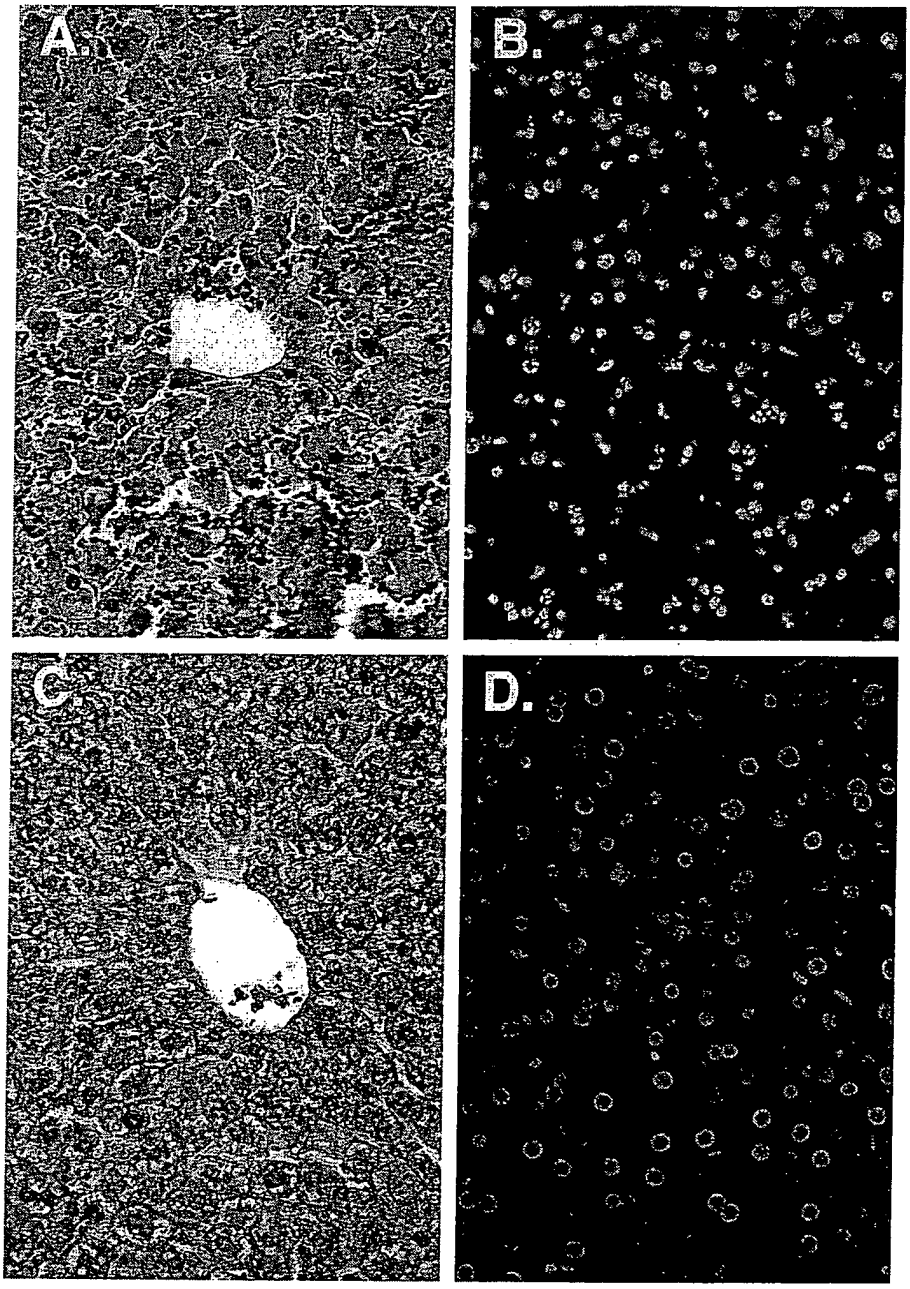

FIG. 7. In situ detection of apoptotic cells in the liver. Adult female C57BL/6 mice were injected intravenously with AdLoxpFasL plus AxCANCre (A and B) or AdCMVLacZ (C and D). Livers were harvested at 24 h postinjection, and sections were prepared for histological analysis staining with hematoxylin and eosin (A and C) or with Hoechst 33258 reagent to detect apoptosis (B and D). Magnification, ×320.

the host immune response to the vector-transduced cells (2, 41). When this experiment was repeated except with in situ induction of mFasL, the pattern of transgene expression did not differ from that noted in the noninduced group (Fig. 8); a rapid reduction in transgene expression was noted such that by day 50 postinjection, a decrement of more than 3 orders of magnitude was noted. Of note, analysis of the infected muscle sites by reverse transcription-PCR confirmed expression of mFasL in the induced group. In addition, in the noninduced group, lower levels of mFasL could be detected (data not shown). Thus, autocrine suicide of infected muscle cells (negative Fas receptor) expressing mFasL was not likely the basis of diminishing transgene expression in the group with induction of mFasL. It thus appeared that in this organ context, simple ectopic expression of mFasL did not achieve the desired end of establishing the vector-infected cell as an immunologically privileged site.

A recent report by Muruve et al. has also described the construction and characterization of a recombinant adenovirus vector expressing mFasL (32). Many of their findings are of

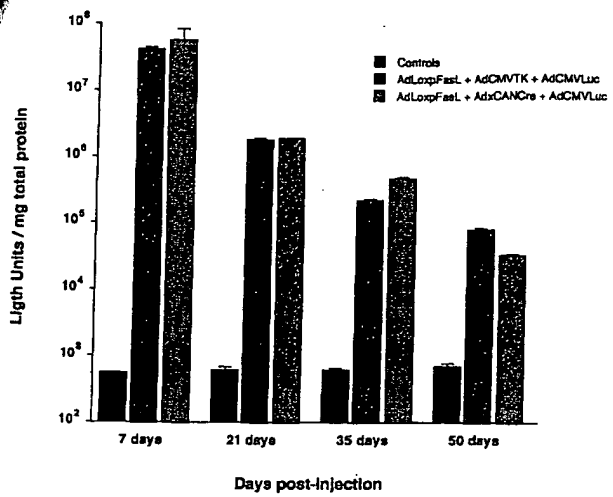

FIG. 8. Effect of ectopic expression of mFasL expression on longevity of transgene expression in murine myocytes. Adult female BALB/c mice were injected intraglossally with the indicated vectors; control animals were not injected. At various times postinjection, luciferase activity was determined in harvested tissue. Each histogram represents the mean ± standard deviation for seven animals.

interest in the context of the results of this study. These investigators used a cytomegalovirus-driven mFasL cassette in their adenovirus vector. For this construction, they experienced difficulty deriving the virion and have noted consistently low viral titer yields. This was attributed, in part, to the fact that the adenovirus packaging cell line 293 is known to express Fas (32). Thus, the resulting autocrine loop has confounded their vector derivation and propagation efforts. Anticipation of this issue led us to develop an adenovirus vector with an inducible system. In this manner, we have readily obtained the desired recombinant vectors and obtained viral titers commensurate with those of standard recombinant adenovirus vectors. Thus, from a strictly practical standpoint, we have derived a benefit from maintaining the mFasL in an inducible state in the context of the adenovirus vector. An additional aspect of the report of Muruve et al. was the finding that systemic injection of their vector induced widespread death of hepatocytes, a phenomenon consistent with the effects of anti-Fas antibody (38). Furthermore, transduced pancreatic allografts underwent apoptotic cell death, resulting in nonfunctional grafts when transplanted into syngeneic or allogenic recipients. The latter phenomenon is significant, as Fas is widely expressed. Whereas Muruve et al. did not explicitly use FasL to prolong transgene expression in cells infected by adenovirus vectors, their results did provide insight into the complexity of the Fas-FasL pathway. In this context, Allison et al. (4) reported that expression of functional FasL in the pancreatic islets of transgenic mice failed to protect these islets from allogenic transplant rejection. In addition, these genetically modified cells induced a granulocytic infiltrate that damaged the islets (4, 8, 23). Further of note, Seino et al. reported that FasL expression in tumor cells could induce a granulocyte-mediated rejection (48). A further new study challenges the immunoprotective effect of FasL; Kang et al. have shown that adenovirus-mediated expression of FasL in pancreatic islet allografts induces neutrophilic infiltration and islet destruction (23). One more example of the complexity of the Fas-FasL system is the discovery that various disease states result from dysregulation of the system, including lymphoproliferative autoimmune syndromes, hepatitis, Hashimoto's thyroiditis, and glomerular cell apoptosis (11, 13). Finally, Kayagaki et al. observed that naturally occurring alleles of FasL have different abilities to trigger apoptosis through Fas, suggesting that polymorphism of FasL affects the biological activity (24).

These results parallel the limitations of our study in that ectopic FasL expression per se was not sufficient to prolong expression of a vector-encoded transgene by attempting to diminish immunological eradication of vector-transduced cells. Thus, several issues with respect to the use of FasL have arisen in these studies. First, while intended to allow an immunologically privileged site, the ectopic expression of FasL can actually elicit an inflammatory influx. Second, the coexpression of Fas and an ectopic FasL can induce an autocrine loop with induction of target cell apoptosis. In addition, the magnitude and temporal pattern of FasL expression may be key determinants of its efficacy in this context. These issues were not addressed in our study. The complex aspects of FasL biology can confound direct attempts to explain this axis in many tissue contexts and can be frankly deleterious at some organ sites through elicitation of parenchymal apoptosis. Thus, a more complete understanding of the Fas-FasL pathway will be required before strategies to exploit the system for mitigating vector immunogenicity may be contemplated.

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Mature But Not Immature Fas Ligand (CD95L)-Transduced Human Monocyte-Derived Dendritic Cells Are Protected from Fas-Mediated Apoptosis and Can Be Used as Killer APC[1]

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Several in vitro and animal studies have been performed to modulate the interaction of APCs and T cells by Fas (CD95/Apo-1) signaling to delete activated T cells in an Ag-specific manner. However, due to the difficulties in vector generation and low transduction frequencies, similar studies with primary human APC are still lacking. To evaluate whether Fas ligand (FasL/CD95L) expressing killer APC could be generated from primary human APC, monocyte-derived dendritic cells (DC) were transduced using the inducible Cre/Loxp adenovirus vector system. Combined transduction of DC by AdLoxpFasL and AxCANCre, but not single transduction with these vectors, resulted in dose- and time-dependent expression of FasL in >70% of mature DC (mDC), whereas <20% of immature DC (iDC) expressed FasL. In addition, transduction by AdLoxpFasL and AxCANCre induced apoptosis in >80% of iDC, whereas FasL-expressing mDC were protected from FasL/Fas (CD95/Apo-1)-mediated apoptosis despite coexpression of Fas. FasL-expressing mDC eliminated Fas$^+$ Jurkat T cells as well as activated primary T cells by apoptosis, whereas nonactivated primary T cells were not deleted. Induction of apoptosis in Fas$^+$ target cells required expression of FasL in DC and cell-to-cell contact between effector and target cell, and was not dependent on soluble FasL. Induction of apoptosis in Fas$^+$ target cells required expression of FasL in DC, cell-to-cell contact between effector and target cell, and was not dependent on soluble FasL. The present results demonstrate that FasL-expressing killer APC can be generated from human monocyte-derived mDC using adenoviral gene transfer. Our results support the strategy to use killer APCs as immunomodulatory cells for the treatment of autoimmune disease and allograft rejection. *The Journal of Immunology,* **2003, 170: 5406–5413.**

Dendritic cells (DC)[3] are a heterogeneous population of bone marrow-derived cells present in most peripheral tissues that are able to capture and present Ags to the cells of the adaptive immune system (1). Such antigenic presentation to T cells can lead to two opposite outcomes: potent activation (immunogenicity); or inhibition (tolerance) of effector immune functions (2, 3). Recent progress in understanding of the physiological function of DC and technological advances in generating large numbers of DC from various progenitors in vitro have led to the development of many DC-based vaccines as a potent strategy to initiate protective immune responses against tumors and infectious pathogens. In contrast, novel strategies were developed to enhance the capacity of DC to induce immunological tolerance. These strategies include pretreatment of DC with IL-10 (4), UV irradiation (5), use of DC in an immature state (6), or transduction of DC with immunoregulatory molecules such as IL-10 (7), TGF-β (8), and CTLA-4 (9).

One central mechanism of immune privilege is use of the Fas/Fas ligand (FasL)-mediated apoptosis to delete invading T cells at immune privilege sites, and constitutive production of FasL has been demonstrated by Sertoli cells of the testes and the retinal cells of the eye (10). In addition, expression of FasL has been observed in different tumor cells suggesting that FasL/Fas-mediated apoptosis might be a critical mechanism by which tumors evade the immune response as proposed in the tumor counterattack model (11). With regard to these observations, studies were performed to modulate the interaction of APCs and T cells by triggering Fas apoptosis signaling, which should result in deletion of activated T cells as proposed for immune privilege tissues.

Ag-specific elimination of activated T cells by FasL-expressing APC has been demonstrated by us and other investigators in different in vitro and in vivo experimental models using murine cells or cell lines as APC. These encouraging results indicated the therapeutic potential of FasL-expressing killer APC as immunoregulatory cells for the treatment of allograft rejection (12–16), autoimmune disease (17–21), and chronic infections (22, 23). However, it has not been determined whether human DC can be efficiently transduced by FasL and whether these cells are capable to eliminate Fas$^+$ target cells. In contrast to murine cells or cell lines, transduction of human monocyte-derived macrophages and DC is difficult, and high transduction rates could be achieved only with viral gene transfer strategies (24). In addition, in previous studies, FasL-expressing APC were generated from mice deficient in Fas-mediated apoptosis to prevent self-destruction of transduced FasL-expressing APC. Fas-mediated self-destruction also hampers the propagation of FasL-encoding viral vectors in Fas$^+$ host cells

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[3] Abbreviations used in this paper: DC, dendritic cell; FasL, Fas ligand; iDC, immature DC; mDC, mature DC; MOI, multiplicity of infection; sFasL, soluble FasL; PI, propidium iodide; AdEGFP, recombinant adenovirus encoding EGFP.

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(25). Therefore, experiments to generate FasL-expressing APC from primary human cells are still lacking. Because several studies demonstrated that FasL is highly conserved within different species and that murine FasL induces apoptosis in human Fas^+ cells (18), we investigated in the present study whether primary human monocyte-derived immature DC (iDC) and mature DC (mDC) could be transduced with murine FasL using an inducible Cre/Loxp adenovirus-based gene transfer system to generate killer APC.

Materials and Methods

Preparation and culture of monocyte-derived DC, primary T cells, and Jurkat T cells

PBMC were isolated from leukapheresis concentrates of healthy donors, and monocytes were separated by countercurrent elutriation (26). To induce the in vitro differentiation of monocytes to DC, monocytes were cultured for 7 days in serum-free CellGro culture medium (CellGenix, Freiburg, Germany) in the presence of 500 U/ml IL-4 (Promocell, Heidelberg, Germany) and 500 U/ml GM-CSF (Leukomax; Essex, Munich, Germany). To induce maturation of DC, cells were additionally stimulated with IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-6 (1000 U/ml), all from Promocell (Heidelberg, Germany) and PGE_2 (1 μg/ml; Minprostin E_2; Pharmacia & Upjohn, Erlangen, Germany) for an additional 2 days as described previously (27). To confirm the phenotypes of iDC and mDC, expression of the markers CD83, CD80, CD86, and HLA-DR was determined in cultured DC by FACS analysis.

T cells were separated by countercurrent elutriation at a flow rate of 52 ml/min in HBSS and 6% autologous plasma. Cells were frozen immediately in RPMI culture medium containing 10% DMSO (Sigma-Aldrich, Steinheim, Germany) and 40% heat-inactivated (56°C for 30 min) autologous plasma. For additional experiments, T cells were rapidly thawed at 37°C, washed with PBS (Life Technologies Invitrogen, Paisley, U.K.), and resuspended in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 200 mM L-glutamine, 100 mM sodium pyruvate, non-essential amino acids, minimal essential medium vitamins (all from Life Technologies), penicillin/streptomycin (50 U/50 μg/ml; PAA Laboratories, Linz, Austria) and 50 μM 2-ME (Amresco, Solon, OH). For polyclonal activation, aliquots of T cells were stimulated with PHA (1 μg/ml) for 5 days.

Jurkat T cells (purchased from DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all from PAA), 1 mM HEPES, and 1 μg/ml amphotericin B (both from Sigma-Aldrich, Deisenhof, Germany).

Recombinant adenoviruses and transduction of DC

To prevent Fas/FasL-mediated apoptosis during virus propagation in Fas^+ 293 cells and to facilitate inducible transgene expression, recombinant adenoviruses encoding murine full-length FasL were generated using the Cre/Loxp-inducible system. AdLoxpFasL was generated by separating the *FasL* gene from the modified chicken β-actin promoter with the CMV immediate-early enhancer by a Loxp-flanked stuffer segment as previously published. The Cre recombinase protein encoded in AxCANCre has been demonstrated to excise the stuffer and allow functional reconstitution of the expression cassette at the deleted E1 site of the recombinant adenovirus vector leading to FasL expression in cells simultaneously transduced with AxCANCre and AdLoxpFasL (28, 29). A recombinant adenovirus encoding EGFP (AdEGFP) was used as a control vector. Viruses were propagated in HEK 293 cells (Clontech, Heidelberg, Germany) and enriched by ultracentrifugation as described (30).

For transduction, iDC and mDC were used 9 days after initiation of cultures. According to a previously published protocol (31), DC were incubated at a concentration of 2×10^6/ml in serum-free CellGro culture medium for 90 min with or without the different recombinant adenoviruses. Afterward, cells were resuspended at a concentration of 1×10^6/ml by addition of fresh culture medium containing 500 U/ml IL-4 as well as 500 U/ml GM-CSF. A multiplicity of infection (MOI) of 200 was determined as optimal and used throughout additional experiments for single transductions, and a MOI of 100 for each vector was used in double-transduction experiments. On day 12 (3 days after transduction), untreated and transduced DC were used for additional experiments. For time course and dose dependency experiments, cells were transduced with different MOIs as indicated and analyzed on day 1, 2, or 3 after transduction.

RT-PCR analysis for FasL mRNA

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions involving an additional DNA digestion step for 30 min (RNase-free DNase Set; Qiagen). Total RNA was used to synthesize cDNA using the 1st Strand cDNA Synthesis Kit including avian myeloblastosis virus for reverse transcription (Roche Diagnostics, Mannheim, Germany). RT-PCR for the murine FasL was performed using the forward primer 5′-AGGAATGTATACGCTCTTCC-3′ and the reverse primer 5′-CCTCATATAGACCTTGTGGT-3′ (product 369 bp). Primers for amplification of 18s were forward 5′-TCAAGAAC GAAAGTCGGAG-3′ and reverse 5′-GGACATCTAAGGGCATCACA-3′ (product 488 bp). RT-PCR was performed in a 20-μl reaction volume containing the *Taq* PCR Master Mix Kit (Qiagen), 10 pmol from each primer of a primer pair, and 2 μl of cDNA. All samples were incubated for 20 cycles (denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 30 s at 72°C) using a thermocycler (GeneAmp PCR System 9700; PE Applied Biosystems, Foster City, CA). RT-PCR products were separated on a 1% agarose gel (FCM Bioproducts, Rockland, ME) and visualized by GelStar staining (BioWhittaker Molecular, Rockland, ME).

FACS and apoptosis detection

After two washings with PBS containing 10% FCS (PAA), cells were stained for murine FasL on the surface (PE-conjugated, clone Kay-10) or isotype control PE-conjugated mouse anti-mouse IgG2b (both purchased from BD PharMingen, San Diego, CA). After incubation for 45 min on ice in the dark followed by two washing steps, cells were fixed in PBS containing 0,1% paraformaldehyde (Sigma-Aldrich), and cells were analyzed using a EPICSXL-MCL (Coulter Electronics, Miami, FL). For detection of endogenous expression of human Fas and human FasL, DC were stained with PE-conjugated anti-Fas (clone VI C-64), biotin-conjugated anti-FasL (clone NOK-1) in combination with PE-conjugated streptavidin, or isotype control Abs (PE-conjugated anti-mouse IgG1, all purchased from BD PharMingen).

For detection of apoptosis, cells were washed twice with ice cold PBS and stained simultaneously with FITC-conjugated annexin V and propidium iodide (PI) according to the manufacturer's instructions (both from BD PharMingen) for 20 min on ice in the dark with a binding buffer containing 10 mM HEPES-NaOH, 140 mM NaCl, and 2.5 mM $CaCl_2$ (all from Sigma-Aldrich). Within the next hour, cells were analyzed for apoptosis. Total numbers of apoptotic cells were determined by calculation of annexin V^+ and PI^- cells (reflecting early apoptosis) together with annexin V^+ and PI^+ cells (reflecting late apoptosis/secondary necrosis). Analysis of data was performed using the software WinMDI (version 2.8; http://facs.scripps.edu).

Cytotoxicity assay

To determine FasL-mediated cytotoxicity, cocultures of Jurkat T cells or activated and nonactivated primary T cells were established with transduced DC at different E:T ratios. Cytotoxicity was detected either by FACS using annexin V and PI staining as described, or using the JAM assay as previously published (32, 33).

To label the Fas^+ Jurkat T cells as targets for the JAM assay, 5×10^5/ml cells were incubated overnight with 2,5 μCi/ml [*methyl*-^{3}H]thymidine (Amersham Pharmacia, Erlangen, Germany). After two washing steps using PBS, 4×10^4 labeled Jurkat T cells were cocultured with 10^4 FasL-expressing DC or control DC. As a positive control, 4×10^4 labeled Jurkat T cells were incubated with an activating anti-Fas Ab (0.5 μg/ml, clone CH11; Upstate, Charlottesville, VA). To determine dose dependency, different amounts of mDC transduced with AxCANCre and AdLoxpFasL were incubated with 5×10^4 labeled Jurkat cells. To investigate whether cytotoxicity of FasL-expressing DC is cell contact dependent, additional Transwell experiments were performed, in which 4×10^4 labeled Jurkat cells were placed in 96-well microtiter plates, and Transwells (Nunc, Roskilde, Denmark) containing 10^4 FasL-expressing DC were added. After 5 h, cells were lysed, and DNA was transferred onto glass fiber filters (Printed Filtermat B; Wallac Oy, Turku, Finland) using a Vacusafe IH-280 harvester (Innotech, Dottikon, Switzerland). After drying, filters were transferred to scintillation fluid (Betaplate Scint; Wallac U.K., Milton Keynes, U.K.) and counted using a Wallac MicroBeta liquid scintillation counter (1450 MicroBeta; Wallac Oy). Percent apoptosis was quantitated using the following formula: % specific apoptosis = $(cpm_{spontaneous} - cpm_{experimental}/cpm_{spontaneous}) \times 100$. All samples were tested in quadruplicate and are presented in medians with 25–75% interquartiles.

Detection of soluble FasL (sFasL)

On day 3 after transduction, supernatants from the DC cultures were obtained and stored at −20°C. The soluble murine FasL was determined using a specific ELISA (R & D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. Results are indicated as means ± SD obtained from at least three independent experiments.

Results

Expression of FasL after transduction of iDC and mDC by AdLoxpFasL and AxCANCre

To generate killer APC, iDC and mDC were transduced simultaneously by AdLoxpFasL and AxCANCre. Transduction of DC with either AdLoxpFasL, AxCANCre or AdEGFP was served as the controls. RT-PCR analysis revealed an up-regulated expression of FasL-mRNA in DC simultaneously transduced by AdLoxpFasL and AxCANCre, whereas FasL-mRNA was not detected in AdEGFP- or AxCANCre-transduced DC (Fig. 1*A*). As previously observed, FasL-mRNA could be also detected at low levels in AdLoxpFasL singly-transduced DC, suggesting that some spontaneous transgene-expression has occurred using these vectors (34).

To evaluate whether FasL-protein was indeed expressed by AdLoxpFasL and AxCANCre cotransduced DC, FACS-analysis was performed. There was a strong expression of FasL in 80% of mDC 3 days after combined transduction with AdLoxpFasL and AxCANCre. In contrast, FasL could not be detected on the surface of mDC after single transductions by either AdLoxpFasL or AxCANCre. However, <20% of iDC expressed FasL on the surface 72 h after simultaneous transduction with AdLoxpFasL and AxCANCre. These results demonstrated that a combined transduction of these vectors was required for FasL-expression, and that particularly mDC could be efficiently transduced to express FasL (Fig. 1*B*).

Apoptosis was induced in iDC but not mDC after transduction with FasL

Expression of Fas on the surface of monocyte-derived DC has been described by several investigators (35–38). To analyze whether self-destruction might be induced in DC after transduction with FasL, endogenous expression of Fas and FasL was determined in iDC and mDC by FACS. Consistent with previous reports, Fas was expressed in the majority of iDC and mDC, and there was no difference in the expression levels of Fas between both cell populations. In contrast, endogenous human FasL could be detected on the surface of less than 10% of mDC, whereas endogenous FasL was not detectable in iDC (Fig. 2).

These results suggested that Fas-mediated self-destruction might occur in DC as a consequence of FasL expression, limiting the potential usage of these cells. Therefore, apoptosis was detected in iDC and mDC 72 h after adenoviral transduction by annexin V and PI staining. Total numbers of apoptotic cells were determined by calculation of annexin V^+ and PI^- cells (reflecting early apoptosis) together with annexin V^+ and PI^+ cells (reflecting late apoptosis/secondary necrosis). There was an apoptosis rate of 91% in iDC 72 h after simultaneous transduction with AdLoxpFasL and AxCANCre, whereas <20% of the singly-transduced iDC were apoptotic. In contrast, ~70% of FasL-expressing mature DC remained viable at day 3 after transduction with FasL, demonstrating that mDC were protected from Fas-mediated apoptosis despite coexpression of Fas (Fig. 3*A*).

To determine the pattern of FasL expression and apoptosis induction, time course experiments were performed at days 1, 2, and 3 after FasL transduction using different concentrations of adenoviral vectors (Fig. 3*B*). There was a time- and a dose-dependent expression of FasL in mDC, which peaked 3 days after transduction with AdLoxpFasL and AxCANCre. Expression of FasL or increasing concentrations of adenoviral vectors did not affect apoptosis of mDC, given that the percentage of apoptotic DC was below 10% at all time points tested and virus concentrations used. In contrast, only low levels of FasL were expressed in iDC (<20%) 3 days after transduction using high concentrations of AdLoxpFasL and AxCANCre. In addition, there was a time- and a dose-related induction of apoptosis in iDC. Consequently, the percentage of apoptotic iDC increased above 60% 3 days after FasL transduction using the high virus concentrations.

Efficient elimination of Fas^+ Jurkat T cells by FasL-expressing DC

Next, we sought to analyze the capacity of FasL-expressing DC to induce apoptosis in Fas^+ target T cells. JAM assays were performed using FasL-expressing DC as effector cells and Jurkat cells, a human T cell lymphoma cell line, as target cells. There was a significant increase in apoptosis in 60% of the Jurkat T cells 5 h

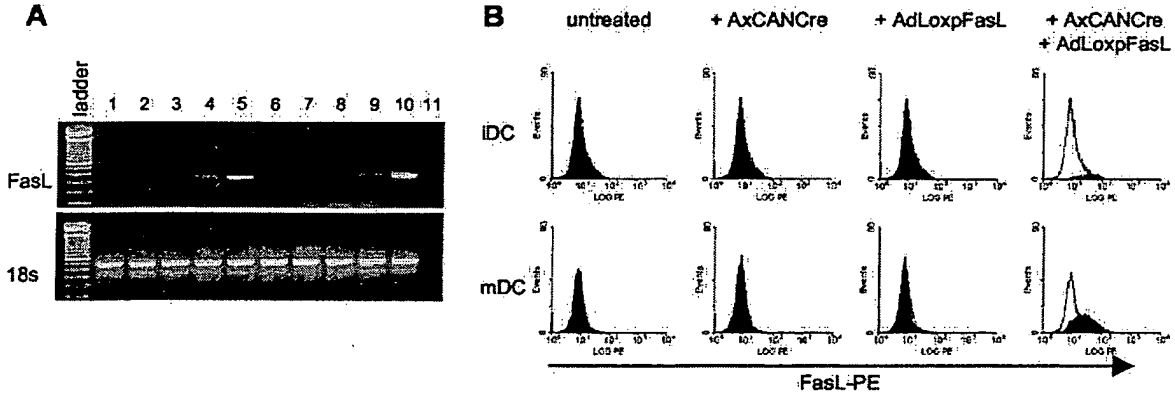

FIGURE 1. *A*, Expression of FasL determined by RT-PCR. iDC and mDC were simultaneously transduced by AdLoxpFasL and AxCANCre using a MOI of 100 for each vector. Single transductions were performed with AdEGFP, AxCANCre, or AdLoxpFasL as controls using a MOI of 200 for each vector. Expression of FasL-mRNA was determined by RT-PCR (product, 369 bp). The expression of 18S was used as the control (product, 488 bp). *Lane 1*, iDC untreated; *lane 2*, iDC plus AdEGFP; *lane 3*, iDC plus AxCANCre; *lane 4*, iDC plus AdLoxpFasL; *lane 5*, iDC plus AxCANCre/AdLoxpFasL; *lane 6*, mDC untreated; *lane 7*, mDC plus AdEGFP; *lane 8*, mDC plus AxCANCre; *lane 9*, mDC plus AdLoxpFasL; *lane 10*, mDC plus AxCANCre/AdLoxpFasL; *lane 11*, RT-PCR H_2O control. *B*, Expression of FasL determined by FACS. Three days after transduction of iDC and mDC, expression of murine FasL was analyzed by staining the cells with a mAb (filled curves), or the isotype control (open curves). Results are representative of five independent experiments.

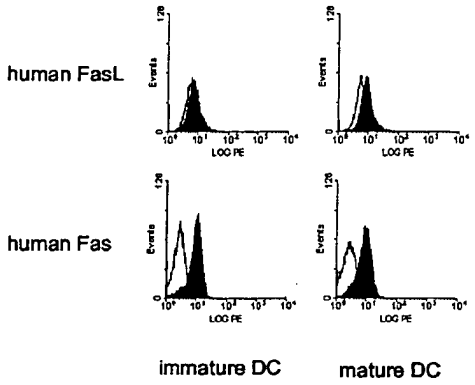

FIGURE 2. Endogenous expression of human Fas and FasL by iDC and mDC. Immature DC and mDC were stained on day 9 with mAbs directed against human Fas and FasL (filled curves), or the isotype controls (open curves).

after initiation of cocultures with FasL-expressing mDC, whereas there was no increase in apoptosis of Jurkat T cells after coculture with AdEGFP-, AxCANCre-, or AdLoxpFasL-transduced DC lacking murine FasL. The rate of apoptosis induced by FasL-expressing mDC was equivalent to that observed by treatment of Jurkat T cells with an activating anti-Fas Ab. In contrast, iDC exhibited a reduced capacity to induce apoptosis in Jurkat T cells compared with mDC, because apoptosis was detected in <10% of Jurkat T cells cocultured with iDC transduced by AdLoxpFasL and AxCANCre (Fig. 4*A*).

In addition, cultures of FasL-expressing mDC and Jurkat T cells were established at different E:T ratios, and cytotoxicity was detected using the JAM assay. There was an apoptosis rate of >80% in Jurkat T cells at the E:T ratio of 1:1 demonstrating the exceptional capacity of mDC to induce apoptosis in Fas^+ target cells (Fig. 4*B*). These results were confirmed by FACS using annexin V and PI staining demonstrating apoptosis in 33% of Jurkat T cells cocultured for 5 h with FasL-expressing DC at E:T 1:5 (Fig. 4*C*).

Efficient elimination of activated but not nonactivated primary T cells by FasL-expressing DC

To analyze whether also primary T cells could be eliminated by FasL-expressing DC, additional cytotoxicity experiments were performed. Cocultures of PHA-activated primary T cells and nonactivated primary T cells were established with FasL- or EGFP-transduced mDC at E:T 1:5, and numbers of apoptotic T cells were determined after 5 h by annexin V and PI staining. FACS revealed apoptosis in 47% of the PHA-activated T cells cocultured with FasL-expressing DC, whereas apoptosis could be observed in 14% of PHA-activated T cells cocultured with EGFP-transduced DC. In contrast, apoptosis was not induced in nonactivated T cells cocultured with either FasL- or EGFP-expressing DC. These results indicated that activation of primary T cells was required for induction of apoptosis by FasL-expressing killer DC (Fig. 5).

Induction of apoptosis in Fas^+ target T cells required contact with FasL-expressing DC and was not dependent on sFasL

To analyze whether induction of apoptosis in Fas^+ target cells by FasL-expressing DC might be affected by sFasL, concentrations of murine sFasL were determined in supernatants of transduced DC using a specific ELISA. There were high levels of sFasL above 3000 pg/ml in culture supernatants of mDC and iDC 72 h after transduction with AdLoxpFasL and AxCANCre, whereas only low concentrations (<25 pg/ml) of sFasL could be detected in supernatants of cultures containing DC after single transduction with these vectors or control cultures containing untreated DC (Fig. 6).

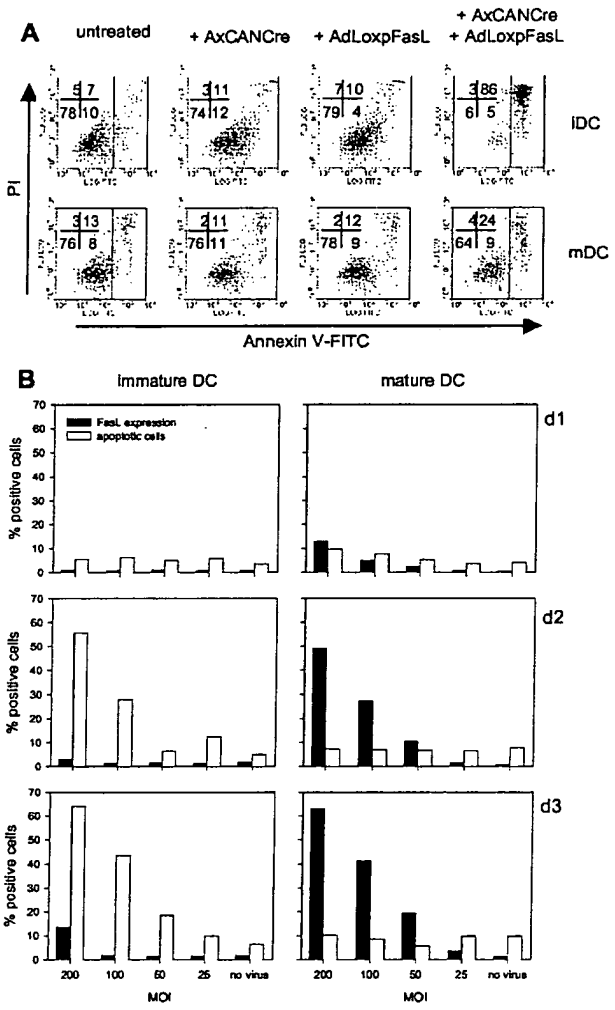

FIGURE 3. *A*, Induction of apoptosis in iDC but not mDC after transduction with FasL. Three days after transduction, iDC and mDC were stained with annexin V-FITC and PI for detection of apoptosis. One of five experiments is depicted. *B*, Time- and dose-dependent expression of FasL and induction of apoptosis in iDC and mDC. After transduction of iDC or mDC with AxCANCre and AdLoxpFasL at different MOIs, the cells were analyzed for FasL expression and apoptosis induction. □, Percent of cells positive for the murine FasL; ■, percent of apoptotic DC (annexin V^+PI^- and annexin V^+PI^+). One of three independent experiments is depicted.

These results suggested that sFasL could be involved in apoptosis induced by FasL-expressing DC; therefore, additional Transwell experiments were performed to test this hypothesis. However, there was no increase in apoptosis in Jurkat T cells cocultured with FasL-expressing mDC when these cell populations were separated by a membrane. In contrast, apoptosis could be induced in 40% of Jurkat T cells by an activating Fas Ab, a positive control that can efficiently induce apoptosis in Jurkat T cells in a soluble form. These results demonstrated that elimination of Fas^+ target cells by FasL-expressing killer APC required cell-to-cell contact and was not related to production of sFasL (Fig. 7).

Discussion

The results obtained in this study provide novel information that human monocyte-derived mDC, but not iDC, can be efficiently

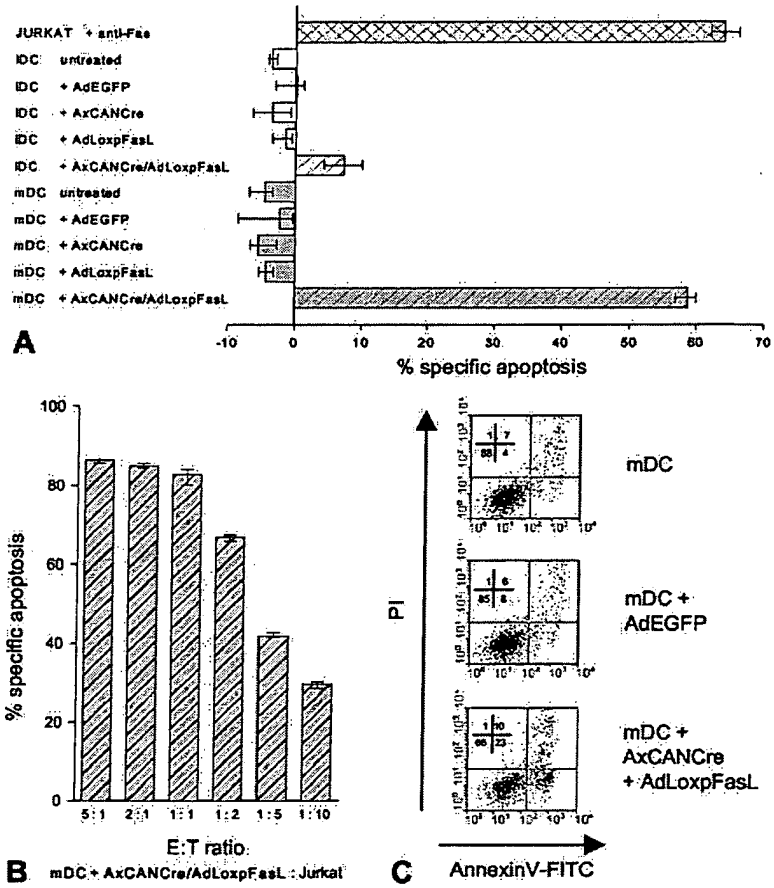

FIGURE 4. *A*, Cytotoxicity of FasL-expressing DC. Immature DC (□) and mature DC (▨) were transduced with AdEGFP, AxCANCre, or AdLoxpFasL or simultaneously with AxCANCre and AdLoxpFasL as indicated. After 72 h, cocultures of FasL-expressing DC (gray and white hatched bars) and control DC were established with Fas$^+$ Jurkat T cells labeled with [*methyl*-^{3}H]thymidine (E: T 1:4). As a control, labeled Jurkat cells were treated with an activating anti-Fas Ab (clone CH11 0, 5 μg/ml, ▩). Specific apoptosis was detected using the JAM assay as described in *Materials and Methods*. The depicted experiment is representative of three independent experiments. *B*, Cocultures of FasL-expressing mDC and [*methyl*-^{3}H]thymidine-labeled Jurkat T cells were established at different E: T ratios, and apoptosis was detected 5 h later using the JAM assay. *C*, The apoptosis-inducing capacity of FasL-expressing DC was confirmed by staining Jurkat T cells with annexin V and PI 5 h after initiation of cocultures (E:T 1:5). One of three independent experiments is depicted.

transduced using adenoviral vectors to express high levels of FasL. These FasL-expressing mDC might act as killer APC to eliminate Fas$^+$ target cells including activated primary T cells in a cell-to-cell contact-dependent manner. Thus, the capacity to modify immune responses through FasL-expressing killer APC would provide a significant potential for improvement of therapies for a number of T cell-dependent diseases such as autoimmune diseases, allograft rejection, and chronic infections.

Ag-specific deletion of activated T cells by FasL-expressing APC has been reported previously by us and other investigators. However, all these studies were performed using murine DC, B cells, macrophages, or cell lines as APC (12–23, 39). Therefore, for future clinical application, it had to be determined whether human DC can be efficiently transduced with FasL and whether these cells would be capable of eliminating Fas$^+$ target cells. Phenotypical and functional characterization of DC revealed that DC represent a heterogeneous cell population with diverse functions. These differences have been related to the state of maturation, preparations of DC from different anatomical compartments, and

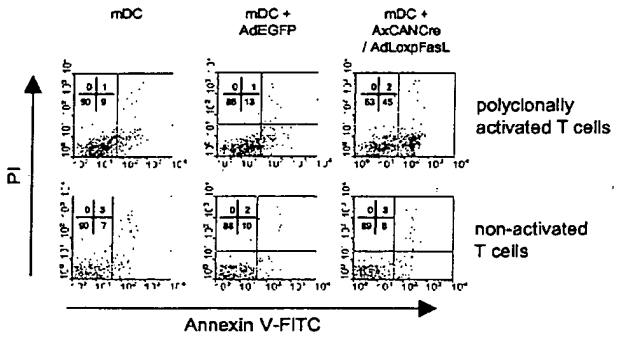

FIGURE 5. Elimination of activated primary T cells by FasL-expressing DC. Cocultures of PHA-activated primary T cells and nonactivated primary T cells were established with FasL- or EGFP-transduced mDC at E:T 1:5, and numbers of apoptotic T cells were determined after 5 h by annexin V and PI staining. The results are representative of four independent experiments.

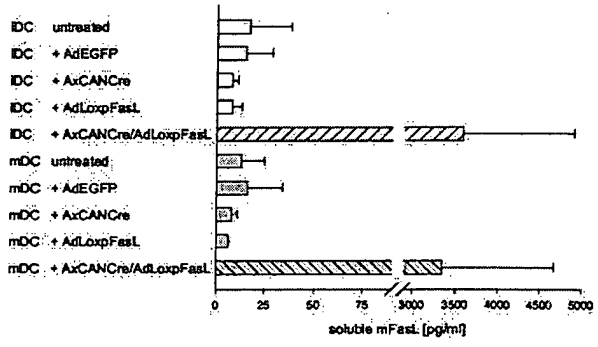

FIGURE 6. Determination of the soluble FasL in supernatants of FasL-expressing DC. Supernatants were obtained 72 h after single transduction of iDC (□) and mDC (▨) with AxCANCre, AdLoxpFasL, and AdEGFP or double transduction of iDC and mDC with AxCANCre and AdLoxpFasL (hatched bars). Levels of murine sFasL were determined using a specific ELISA, and means ± SD of four experiments are depicted.

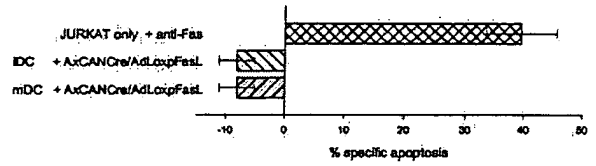

FIGURE 7. Cytotoxicity of FasL-expressing DC required cell-to-cell contact. To determine whether induction of apoptosis by FasL-expressing iDC (white hatched bar) or mDC (gray hatched bar) was dependent on cell-to-cell contact between DCs and Jurkat T cells, the JAM assay was repeated using a Transwell system. FasL-expressing iDC or mDC (10^4) were placed into Transwells, and 4×10^4 labeled Jurkat T cells were seeded into a microtiter plate. As a control, an activating anti-Fas Ab (clone CH11 0, 5 μg/ml, cross-hatched bar) was added into the Transwells. Specific apoptosis was determined using the JAM assay as described in *Materials and Methods*.

generation of DCs using different species. Because viral gene transfer strategies are required to achieve high transduction rates in human but not murine DC (24), it is particularly important that the differences between murine and human DC populations are also reflected in the capacity to express transduced genes (40, 41).

Adenoviral vectors efficiently transduce human iDC and mDC (24, 42). However, propagation of recombinant adenoviruses encoding FasL has been hampered by induction of self-destruction in host cells leading to low virus titers (25). Therefore, the Cre/Loxp adenovirus vector system was used to enable inducible expression of FasL as previously described (29, 34). Using this adenovirus system, we were able to show that expression of high levels of FasL could be achieved in human mDC in a time- and a virus dose-dependent manner. Highest numbers of FasL-expressing mDC could be detected 3 days after adenoviral transduction and remained high up to day 10 (data not shown). These results are consistent with our previous reports demonstrating that FasL could be induced in murine APC using these adenoviral vectors (22, 28). However, gene transfer strategies to induce tolerance based on viral vectors are limited to in vitro studies as virally transduced DC will present viral Ags on the cell surface leading to activation of virus specific T cells. Thus, if these DC coexpress FasL, virus-specific T cells could be eliminated by Fas-mediated apoptosis leading to suppression of the immune response if the organism is infected with the native virus. Therefore, new methods for high level transduction of human DC are required which are not based on viral vectors.

In contrast to mDC, transduction of iDC with AdLoxpFasL and AxCANCre was less effective. This result was surprising because it has been reported that higher transduction rates could be achieved by adenoviral vectors in iDC compared with mDC (24, 42). However, reduced numbers of iDC were recovered after transduction by AdLoxpFasL and AxCANCre but not control vectors suggesting that iDC were efficiently transduced to express FasL but also were rapidly eliminated by FasL/Fas-mediated apoptosis. This concept was supported by our time course experiments demonstrating that there was a time-dependent increase in apoptotic iDC, which were first annexin V^+ and PI^- and became later annexin V^+ and PI^+. The increase in apoptotic iDC was accompanied by an increase in FasL expression in transduced iDC and was also clearly dependent on the dose of AdLoxpFasL and AxCANCre used in these experiments. Together, these results indicated that iDC were rapidly eliminated by Fas-mediated apoptosis shortly after onset of FasL expression, whereas efficiently transduced mDC were protected from apoptosis despite coexpression of Fas and FasL. Therefore, regulatory mechanisms inhibiting the Fas apoptosis signal must be operative downstream of the Fas level in mDC but not iDC. These results are in accordance with previous studies demonstrating that treatment of mDC with activating anti-Fas Abs did not induce apoptosis in these cells despite abundant expression of Fas on the cell surface (37, 43). The resistance of mDC toward Fas-mediated apoptosis has been attributed to the increased expression of anti-apoptotic molecules during DC maturation, and increased levels of c-FLIP and Bcl-x_L have been detected in mDC compared with iDC (35, 38). To prevent Fas-mediated self-destruction, it has been proposed that APC should be transduced with an apoptosis inhibitor in combination with FasL, and protection from Fas-mediated apoptosis could be achieved in murine APC by coexpression of a truncated Fas-associated death domain protein together with FasL using a recombinant vaccinia virus (20). However, our results demonstrate that coexpression of an apoptosis inhibitor is not required if mDC were used for generation of FasL-expressing human killer APC.

It has been reported that human DC are able to directly and effectively mediate apoptotic killing against a wide array of cultured and freshly isolated cancer cells without harming normal cells (44, 45). This antitumor activity has recently been attributed to expression of multiple cytotoxic TNF family ligands including FasL (46). Using adenoviral vectors encoding murine FasL, we were able to discriminate between expressions of endogenous and transduced FasL. FACS revealed small numbers of mDC expressing low levels of endogenous FasL, whereas endogenous FasL could not be detected in iDC. As determined by coculture experiments, untreated iDC or mDC as well as iDC and mDC transduced with control vectors did not exert any detectable cytotoxic activity when Jurkat T cells or activated primary T cells were used as target cells. However, transduction of mDC with AdLoxpFasL and AxCANCre resulted in strong expression of murine FasL, and Fas-mediated apoptosis was efficiently induced in 60% of Jurkat T cells 5 h after initiation of cocultures with transduced FasL-expressing DC as detected by the JAM assay. It is particular important that only a low E:T ratio was required to eliminate the majority of Jurkat T cells, demonstrating the exceptional potency of transduced FasL-expressing DC to act as killer APC, which has been confirmed by staining of target cells with annexin V and PI. Additional cytotoxicity experiments revealed that also primary T cells could be deleted by FasL-expressing DC. However, activation of T cells before establishment of cocultures was required for induction of FasL/Fas-mediated apoptosis, which supported the concept of using killer APC as immunoregulatory cells.

FasL is a type II transmembrane molecule that can be cleaved by specific metalloproteinases to release a 26-kDa soluble form of the protein (47). There are conflicting results regarding the biological role of sFasL. It has been demonstrated that sFasL is able to antagonize the functional activity of membrane FasL (48, 49), whereas other investigators reported that Fas-mediated apoptosis was induced by sFasL in Fas^+ target cells (50). In contrast to human sFasL, mouse sFasL does not appear to be cytotoxic, although a soluble protein corresponding to the entire extracellular domain of murine FasL has been produced experimentally that does induce apoptosis (51). In addition, the significance of sFasL released by APC transduced with FasL has not been determined. The present results demonstrated that high levels of sFasL could be detected in supernatants obtained from iDC and mDC after transduction with AdLoxpFasL and AxCANCre suggesting that sFasL might be involved in apoptosis. To determine the role of sFasL, additional Transwell experiments were performed. These experiments revealed that apoptosis of Fas^+ target cells was not mediated by sFasL, but it required cell-to-cell contact between FasL-expressing DC and Fas^+ target cells. Our results suggest the important role of membrane FasL in this experimental system.

However, it has been reported that the capacity of sFasL to induce apoptosis is closely related to the type of cell expressing Fas (52); therefore, cells other than Jurkat T cells might be eliminated by sFasL. In addition, it cannot be excluded from these experiments that membrane FasL was at least partially antagonized by sFasL despite the strong killing activity exhibited by AdLoxpFasL- and AxCANCre-transduced mDC. Moreover, it has been reported that sFasL induced a neutrophil-mediated inflammatory response that might further limit the application of FasL-expressing killer APC (53, 54). Due to these disadvantages, strategies must be developed to prevent the release of sFasL by FasL-transduced DC. This could be achieved by generation of novel gene transfer vectors encoding a *FasL* gene lacking the metalloproteinase cleavage sites, as previously demonstrated in transfection experiments performed in tumor cell lines (52, 55).

In summary, our results demonstrate that primary human DC can be efficiently transduced by adenoviral vectors to express FasL. Expression of FasL in iDC resulted in self-destruction, whereas FasL-expressing mDC were protected from Fas-mediated apoptosis. These killer APC were able to delete Fas^+ target cells including activated primary T cells by induction of apoptosis, which required cell-to-cell contact between FasL-expressing DC and target cell, and was not dependent on sFasL. These results support the concept of using human FasL-expressing DC as killer APC for elimination of activated T cells involved in allograft rejection, chronic infections, and autoimmune disease.

Acknowledgments

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cells. However, by convention, cells that display peptides associated with class I MHC molecules to $CD8^+$ T_C cells are referred to as **target cells**; cells that display peptides associated with class II MHC molecules to $CD4^+$ T_H cells are called **antigen-presenting cells** (APCs). This convention is followed throughout this text.

A variety of cells can function as antigen-presenting cells. The distinguishing feature of these cells is their ability to express class II MHC molecules and to deliver a co-stimulatory signal. Three cell types are classified as **professional** antigen-presenting cells: dendritic cells, macrophages, and B lymphocytes. These cells differ in their mechanisms of antigen uptake, in whether they constitutively express class II MHC molecules, and in their co-stimulatory activity:

- Dendritic cells are the most effective of the antigen-presenting cells. Because these cells constitutively express a high level of class II MHC molecules and of co-stimulatory activity, they can activate naive T_H cells.
- Macrophages must be activated by phagocytosing microorganisms before they express class II MHC molecules or the co-stimulatory B7 membrane molecule.
- B cells constitutively express class II MHC molecules but must be activated before they express the co-stimulatory B7 molecule.

Several other cell types, classified as **nonprofessional** antigen-presenting cells, can be induced to express class II MHC molecules or a co-stimulatory signal (Table 10-1).

TABLE 10-1

ANTIGEN-PRESENTING CELLS

Professional antigen-presenting cells
Dendritic cells (several types)
Macrophages
B cells
Nonprofessional antigen-presenting cells
Fibroblasts (skin)
Glial cells (brain)
Pancreatic beta cells
Thymic epithelial cells
Thyroid epithelial cells
Vascular endothelial cells

Many of these cells function in antigen presentation only for short periods of time during a sustained inflammatory response.

Because nearly all nucleated cells express class I MHC molecules, virtually any nucleated cell is able to function as a target cell presenting endogenous antigens to T_C cells. Most often target cells are cells that have been infected by a virus or some other intracellular microorganism. However, target cells can also be altered self-cells such as cancer cells, aging body cells, or allogeneic cells from a graft.

EVIDENCE FOR TWO PROCESSING AND PRESENTATION PATHWAYS

Intracellular (endogenous) and extracellular (exogenous) antigens present different challenges to the immune system. Extracellular antigens are eliminated by secreted antibody, whereas intracellular antigens are most effectively eliminated by cytotoxic T lymphocytes (CTLs). To mediate these responses, the immune system uses two different antigen-presenting pathways: endogenous antigens are processed in the **cytosolic pathway** and presented on the membrane with class I MHC molecules; exogenous antigens are processed in the **endocytic pathway** and presented on the membrane with class II MHC molecules (Figure 10-4).

Early evidence suggesting that class I and class II MHC molecules present antigenic peptides derived from different processing pathways was obtained from experiments with two clones of T_C cells specific for influenza virus. One clone was a typical $CD8^+$, class I–restricted T_C cell, but the other was an atypical $CD4^+$, class II–restricted T_C cell. As discussed in Chapter 3, the association between T-cell function and MHC restriction is not absolute. Indeed, an increasing number of reports have described cross-functional T-cell lines—that is, $CD4^+$, class II–restricted T_C clones, and $CD8^+$, class I–restricted T_H clones. L. A. Morrison and T. J. Braciale analyzed two T_C cell lines: one a typical T_C line that recognized influenza hemagglutinin (HA) associated with a class I MHC molecule and the other an atypical T_C line that recognized influenza HA associated with a class II MHC molecule. These researchers sought to determine whether antigen is processed along different pathways for association with class I or class II MHC molecules. In one set of experiments, target cells that expressed both class I and class II MHC molecules were incubated with infectious influenza virus or with UV-inactivated influenza virus. (The inactivated virus retained its antigenic properties but was no longer capable of replicating

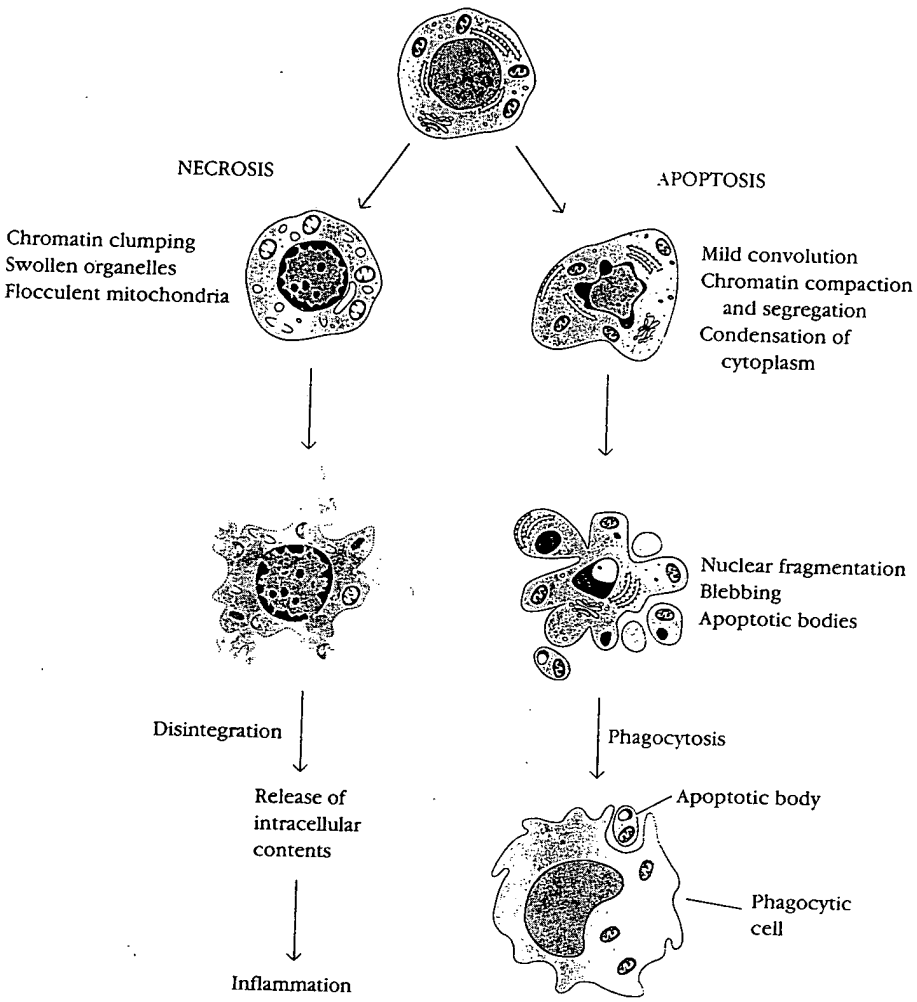

FIGURE 3-4

Comparison of morphologic changes that occur in apoptosis and necrosis. Apoptosis, which is associated with the programmed cell death of hematopoietic cells, does not induce a localized inflammatory response. In contrast, necrosis, the process leading to death of injured cells, results in release of the intracellular contents, which induce a localized inflammatory response.

With fewer receptors on its membrane, the cell becomes progressively less responsive to the CSF, and proliferation of the lineage slows down. This down-modulation of CSF-receptor expression can even be induced by the binding of unrelated CSFs to their receptors. For example, when GM-CSF binds to its receptor, it induces the cell to down-modulate the expression of G-CSF and M-CSF receptors as well. This down-modulation of G-CSF and M-CSF receptors causes the lineages bearing these receptors to become less responsive to these CSFs.

Hematopoiesis can also be regulated by degradation of a CSF following its binding to a receptor. Experiments suggest that binding of M-CSF to its receptor results in degradation of the cytokine. As monocyte numbers increase, there is a corresponding increase in M-CSF receptors, leading to increased M-CSF degradation. Thus the M-CSF concentration falls as cell numbers increase, thereby slowing further proliferation and differentiation of this lineage as long as the number of monocytes remains high.

Programmed Cell Death

In order for steady-state levels of the various hematopoietic cells to be maintained, cell division and differentiation in each of the lineages is balanced by a process called **programmed cell death**. Cells undergoing programmed cell death often exhibit distinctive morphologic changes, collectively referred to as **apoptosis** (Figure 3-4). These changes include a pronounced decrease in cell volume, modification of the cytoskeleton resulting in pronounced membrane blebbing, a condensation of the

chromatin, and degradation of the DNA into oligonucleosomal fragments. Following these morphologic changes, an apoptotic cell sheds tiny membrane-bound apoptotic bodies containing intact organelles. Macrophages quickly phagocytose apoptotic bodies, ensuring that their intracellular contents, including proteolytic and other lytic enzymes, cationic proteins, and oxidizing molecules are not released into the surrounding tissue. In this way apoptosis occurs without inducing a localized inflammatory response. Apoptosis differs markedly from **necrosis**, the changes associated with cell death arising from injury. In necrosis the injured cell swells and bursts, releasing its intracellular contents, which are cytotoxic to other cells in the tissue; as a result, an inflammatory response develops.

Each of the cells produced by hematopoiesis has a characteristic life span and then dies by programmed cell death. In the adult human, for example, there are about 5×10^{10} neutrophils in the circulation. These cells have a life span of only 1 day and then die by programmed cell death. This death, coupled with constant neutrophil production, maintains steady-state levels of these cells. If programmed cell death fails to occur, a leukemic state may develop. Programmed cell death also plays a role in maintaining proper levels of hematopoietic progenitor cells. For example, when colony-stimulating factors are removed, progenitor cells undergo programmed cell death.

The expression of several genes has been associated with the regulation of apoptosis in hematopoietic cell lineages (Table 3-2). Some of these gene products induce apoptosis, whereas other gene products inhibit apoptosis. The *bcl-2* (B-cell lymphoma 2) gene, for example, encodes a protein product that inhibits apoptosis. This gene was originally identified at the breakpoint of a chromosomal translocation in a human B-cell lymphoma. This translocation moved the *bcl-2* gene into the immunoglobulin heavy-chain locus, resulting in transcriptional activation of the *bcl-2* gene and overproduction of the encoded Bcl-2 protein by the lymphoma cells. The resulting high levels of Bcl-2 are thought to contribute to transformation of lymphoid cells into cancerous lymphoma cells by inhibiting the normal signals that would induce apoptotic cell death.

Bcl-2 levels have been found to play an important role in regulating the normal life span of various hematopoietic cell lineages, including lymphocytes. A normal adult has about 5 L of blood with about 2000 lymphocytes/mm^3, for a total of about 10 billion lymphocytes. During acute infection the lymphocyte count increases by 4- to 15-fold, giving a total lymphocyte count of 40-150 billion. Because the immune system cannot sustain such a massive increase in cell numbers for an extended period, the system needs a means to eliminate unneeded activated lymphocytes once the antigenic threat has passed. Activated lymphocytes have been found to express lower levels of Bcl-2 and therefore are more susceptible to apoptotic death than naive lymphocytes or memory cells. If the lymphocytes continue to be activated by antigen, then the signals received during activation bypass the apoptotic signal. As antigen levels subside, so does activation of the lymphocytes and they begin to die by apoptosis (Figure 3-5).

TABLE 3-2

GENES THAT REGULATE APOPTOSIS

GENE	FUNCTION	ROLE IN PROGRAMMED CELL DEATH
bcl-2	Prevents apoptosis	Prevents
bax	Opposes *bcl-2*	Promotes
bcl-X^L (long)	Prevents apotosis	Prevents
bcl-X^S (short)	Opposes *bcl-X*^{L}	Promotes
*ICE**	Protease	Promotes
fas/apo-1	Promotes apoptosis	Promotes

* Gene encoding the interleukin 1β–converting enzyme.

Regulatory Abnormalities and Leukemia

Abnormalities in the expression of hematopoietic cytokines or their receptors may result in some leukemias. Colony-stimulating factors are secreted by a limited number of cells, including activated T lymphocytes, macrophages, endothelial cells, and bone-marrow stromal cells. As mentioned above, each factor induces the proliferation and differentiation of only those hematopoietic stem cells and progenitor cells that bear its receptor. Expression of receptors for a particular growth factor appears to be linked to cellular differentiation following proliferation induced by earlier-acting growth factors. A defect in regulation of expression of either the growth factor or its receptor could lead to unregulated cellular proliferation.

For example, failure to down-modulate receptor expression following GM-CSF activation may lead to a leukemic state. The binding of GM-CSF induces down-modulation of both G-CSF and M-CSF receptors on normal hematopoietic cells but not on leukemic cells (Figure 3-6a, b). This failure of GM-CSF to down-modulate the G-CSF or M-CSF receptors on leukemic cells may allow leukemic cells to respond to low levels of